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Loss of BAF155 impairs neurogenesis in the developing olfactory system of mice

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Abbreviations

| | |
|-----------------------|--|
| Abs | antibodies |
| AG | antigen |
| BAF | Brg1/Brm-associated factor |
| BAF155 ^{-/-} | BAF155 conditional knockout |
| Brg | Brahma-related gene |
| Brm | Brahma |
| Caspase-3 | cysteine-aspartic acid protease |
| cKO | conditional knockout |
| cOE | conditional overexpression |
| DAPI | 4',6'-Diamin-2-phenylindol |
| DEPC | diethyl pyrocarbonate |
| Dil | 1',1'-dihexadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate |
| dcKO | double conditional knockout |
| dKO | double knockout |
| EMC | extracellular matrix |
| esBAF | embryonic stem cell BAF |
| E | embryonic |
| ESC | embryonic ensheating cell |
| FB | forebrain |
| GAP43 | growth associated protein 43 |
| GL | glomeruli |
| GnRH | gonadotropin-releasing hormone |
| HBC | horizontal basal cell |
| IHC | immunohistochemistry |
| IPs | intermediate progenitor cells |
| LOF | loss of function |
| LOT | lateral olfactory tract |
| mAB | monoclonal antibodies |
| Mash1 | mammalian achaete scute homolog-1 |
| Mi | mitral cells |

| | |
|-----------|--|
| MOT | medial olfactory tract |
| M/T cells | mitral/tufted cells |
| mSWI/SNF | mammalian SWItch/Sucrose Non-Fermentable |
| NCAM | neural cell adhesion molecule |
| OB | olfactory bulb |
| OC | olfactory cortex |
| OE | olfactory epithelium |
| OEC | olfactory ensheathing cell |
| OMP | olfactory marker protein |
| ONL | olfactory nerve layer |
| onscBAF | olfactory neuronal stem cell BAF |
| oNSCs | olfactory neuronal stem cells |
| OP | olfactory placode |
| ornBAF | olfactory receptor neuron BAF |
| ORNs | olfactory receptor neurons |
| OSNs | olfactory sensory neurons |
| Otx2 | orthodenticle homeobox 2 |
| pAB | polyclonal antibodies |
| PAC | periamygdaloid complex |
| PBS | phosphate buffered saline |
| PFA | paraformaldehyde |
| pHH3 | phosphorylated Histone H3 |
| Reep6 | receptor expressing-enhancing protein6 |
| Sox2 | sex determining region y (SRY)-box2 |
| SUS | sustentacular cells |
| Tel | telencephalon |
| TFs | transcription factors |
| VNO | vomeronasal organ |

1 Introduction

The olfactory epithelium (OE) enables the gate to the sense of smell- one of the five functions through which human beings and mammals discern the world (Buck 2005). The event of neurogenesis in the olfactory epithelium, which describes the maturing process from olfactory neural stem cells (oNSCs) to olfactory receptor neurons (ORNs) is a vital developmental proceeding, that deserves to move to the center of neurobiological investigation as olfactory receptor neurons, the initial afferent cells of the olfactory epithelium, constitute a cell-type with a unique self-renewing potency throughout lifetime.

The initially mentioned gradual proceeding from oNSC to ORN is known to be directed by a network of transcription factors. However, in which way these transcription factors cooperate with epigenetic and chromatin remodeling systems is still unexplored (Beites et al. 2005; Treloar et al. 2010; Suzuki and Osumi 2015; Bachmann et al. 2016).

Previous studies have already identified chromatin remodeling BAF (Brahma-associated factor) complexes as key factors in the neurogenesis of the central nervous system (Bachmann et al. 2016).

For instance, representative mutations in cerebral cortical volume and thickness have been noticed in studies with cortex-specific BAF170cKO and overexpression (cOE) mice (Tuoc et al. 2013b; Narayanan and Tuoc 2014), as well as the double conditional knockout (dcKO) of the core subunits BAF155 and BAF170 (Nguyen et al. 2016). Thickness, mass and as well surface parameters of the cerebral cortex for instance were enormously increased in BAF170cKO mice, whereas the overexpression of BAF170 resulted in the opposite response in comparison with the wild type animal (Tuoc et al. 2013b; Narayanan and Tuoc 2014).

Nonetheless, the function of mSWI/SNF BAF complexes in the development of olfactory tissues is still under investigation (Bachmann et al. 2016).

Due to the fact, that the function of BAF complexes in the neuronal differentiation of the olfactory system is not yet clarified, we investigated on the consequences of the knockout of the scaffolding core subunit BAF155 in the tissue of the olfactory epithelium.

Current investigations provide evidence, that mSWI/SNF (BAF) complexes constitute a preconditioning element for the unfolding of olfactory neural stem cells to higher differentiated levels (Narayanan and Tuoc 2014).

This finding gave us reason to closer investigate the stepwise differentiation from oNSC to ORN, guided by a special set of transcription factors (TFs).

As above mentioned, it remained to be clarified, how these transcription factors collaborate and interact with the chromatin remodeling systems.

In general, chromatin regulation influences the accessibility of regulatory elements to TFs. In the case of the ATP-dependent BAF complex, by non-covalent, energy-dependent chromatin modulation (Wen et al. 2009; MuhChyi et al. 2013; Ronan et al. 2013; Narayanan and Tuoc 2014).

The relevance of the BAF complex subunit BAF155 becomes obvious by considering the fact, that mice lacking BAF155 (also known as Smarcc1), die in pre- or peri-implantation stages. Further, BAF155 mutants suffer from defects in neural tube closure. Studies propose that these deficiencies might be attributable to a defect in neuronal precursor regeneration and differentiation (Lessard et al. 2007; Wu et al. 2007; Ronan et al. 2013).

To investigate the function of BAF155 in the neurogenesis of the olfactory system, we examined olfactory epithelium-specific BAF155 conditional knockout (cKO) transgenic mice (Bachmann et al. 2016). In this mouse model, the BAF155 knockout is restricted to FoxG1-positive cells. By means of immunohistochemistry (IHC), we studied in which way the loss of BAF155 function (LOF) influences the neurogenic pathway of olfactory neural stem cells.

Data presented in this dissertation is a main part of the published article “mSWI/SNF (BAF) Complexes Are Indispensable for the Neurogenesis and Development of Embryonic Olfactory Epithelium” (Bachmann et al. 2016).

1.1 The olfactory system of mice

This structure is known to be one of the most precocious sensory organs, which develops during early embryogenesis (Treloar et al. 2010).

It includes two components: the peripheral olfactory pathway, which comprises OE and olfactory bulb (OB), and the central olfactory pathway, mainly represented by the olfactory cortex (OC) (Treloar et al. 2010; Suzuki and Osumi 2015; Bachmann et al. 2016).

For the sake of completeness, the vomeronasal organ (VNO) has also to be referred to as being part of the olfactory system. As primarily discovered by Ludvig Levis Jacobson (1783-1843), it is likewise familiar as the Jacobson organ (Zancanaro 2014).

The VNO is placed within the nasal septum of mice, surrounded by a cartilaginous capsule and exhibits glands, ducts, as well as a rich vascular supply. Contrary to the olfactory epithelium, which detects odors, it allows the detection of pheromones from other individuals of the same species and is as that primarily committed to the adjustment of reproductive and defensive properties by means of neuroendocrine secretion (Zancanaro 2014).

Cells of the olfactory system have a long time been considered to evolve merely from the olfactory placode (OP). Contemporary studies, however, gave evidence for a twofold derivation of the olfactory system, namely olfactory placode and neural crest.

Accordingly, the OE emerges from the olfactory placode, which is an area of non-neural ectoderm, whereas the olfactory bulb develops from the neural crest (NC), like numerous central nervous system tissues (Treloar et al. 2010).

The neural crest, formerly reported as “Zwischenstrang” by Wilhelm His in 1868 and later redefined as neural crest by Arthur Milnes Marshall, is situated at the confluence between the dorsal neuroepithelium and epidermis (Douarin and Kalcheim 1999; Achilleos and Trainor 2012; Suzuki and Osumi 2015).

Cells deriving from the NC are multipotent cells and able to drift from their origin to numerous objectives throughout the embryo and process into various cell types (Douarin and Kalcheim 1999; Suzuki and Osumi 2015). Axons from olfactory sensory neurons which are sited in the OE, initially spread in direction of the OB. Here axons establish contact with synapses of excitatory mitral/tufted (M/T) cells in order to constitute the OB glomeruli (López-Mascaraque and Castro 2002; Huilgol and Tole 2016).

Accordingly, in the bulbus olfactorius, the sensory perceptual input of the olfactory epithelium gets connected to the secondary olfactory pathway. Interestingly, cells holding a particular receptor for odors send projections to merely two geographically rooted spots throughout the 1800 glomeruli in the mouse OB (Mombaerts et al. 1996). Amongst the 5 million olfactory sensory neurons (OSNs) situated in the olfactory epithelium of mice, there are 1000-1300 divers

olfactory receptor genes for odors (Malnic et al. 1999; Zhang and Firestein 2002). Main cortical regions in the basal forebrain comprise the olfactory tubercle, the anterior olfactory nucleus (AON), the entorhinal and piriform cortex, as well as several amygdaloid tubercle. These areas are directly innervated by collateral branches of excitatory mitral cells (Mi), located in the OB, via the lateral olfactory tract (LOT) (De Carlos et al. 1996; López-Mascaraque and Castro 2002; Saiz-Sánchez et al. 2011).

This region, called area olfactoria lateralis, predominantly serves as an area where the perception of smell comes to awareness (Trepel 1999).

Projections via the medial olfactory tract terminate in the area olfactoria medialis in the septum region. Here, olfactoric input presumably gets linked to the limbic system (Trepel 1999).

The elements of the olfactory cortex have numerous connections to higher brain structures. The entorhinal cortex for instance passes efferent and receives afferent fibers from the hippocampus throughout its whole length.

The olfactory system is as that unique amongst sensory systems, in passing by pieces of sensory information, without crossing the thalamus (Huilgol and Tole 2016).

The olfactory epithelium, which primarily detects and receives odorants, originates from the olfactory placode that is located in the ventrolateral sides of the head. This process takes place around embryonic (E) day E9.5. This olfactory placode has gone through a thickening process which is governed by environmental and intrinsic stimuli (Suzuki and Osumi 2015).

At day E10.5 the OP invaginates in order to form a nasal cavity (Cuschieri and Bannister 1975; Chen et al. 2009; Forni and Wray 2012; Suzuki and Osumi 2015) which can be visibly detected at E11.5 and has completed invagination at E14.5 (Figure 2). The two components, olfactory placode, respectively olfactory epithelium and olfactory bulb initially develop independently and merge as development continues (López-Mascaraque and Castro 2002). Considering the different origin of these structures, this fact might not come as a surprise. With regard to the dependence of physiologic OB development on the afferent input of OSNs axons, there are still contradicting theories. A prevailing assumption is that the merging together takes place, when early olfactory axons of adult olfactory receptor neurons get in contact with the ventricular zone of the telencepha-

ion at the sensitive phase between E13 and E14. Subsequently, this region starts at E15 to develop the olfactory bulb (Gong and Shipley 1995; Blanchart et al. 2006). The formation of the OB can be classified into these two developmental processes- first induction, later outgrowth (Gong and Shipley 1995; Besse et al. 2011). The outcome of my research reveals, that despite missing axonal afferents of OSNs in BAF155cKO mutants, the forebrain reveals inductional signals.

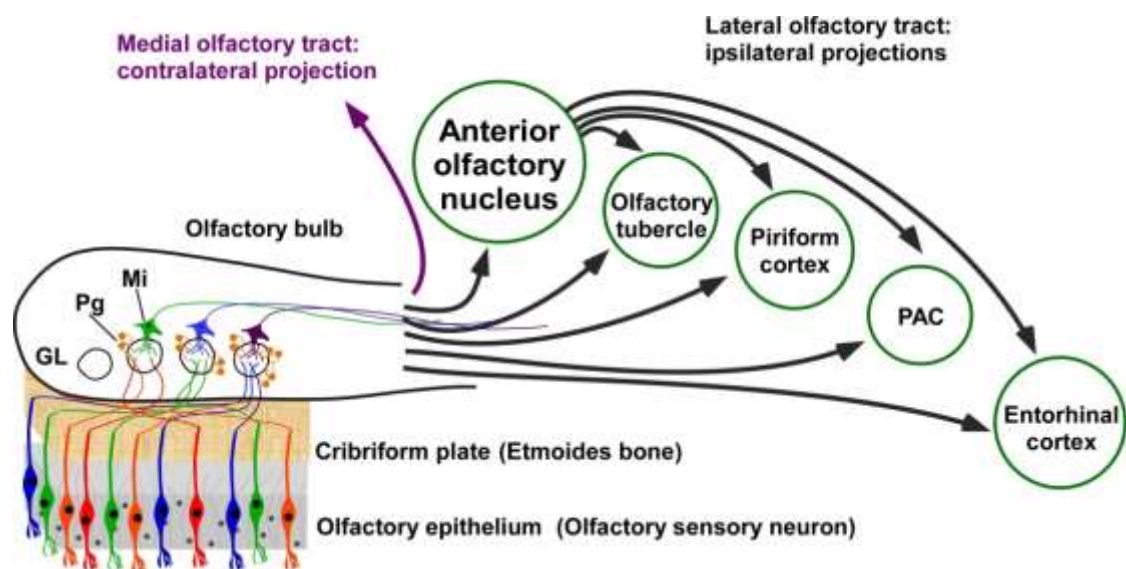


Figure 1: Schematic depiction of the olfactory system

Olfactory sensory neurons send their axons via the cribriform plate to the OB, where they synapse with mitral cells and thus constitute the OB glomeruli (GL). Periglomerular (Pg) inhibitory interneurons as well inhibitory granule cells belong to the compound system of the OB. The collateral branches of mitral cells further project ipsilaterally via the LOT and contralaterally via the medial olfactory tract (MOT) to several cortical regions (black arrows) such as the piriform cortex, olfactory tubercle, anterior olfactory nucleus, periamygdaloid complex (PAC) and entorhinal cortex. The projections via the medial olfactory tract terminate in the septum region of the medial hemisphere. **Source: Saiz-Sánchez et al. 2011**

1.1.1 Development of the olfactory epithelium of mice and cell differentiation

The OE can be classified as a pseudostratified neuroepithelium, that composes various cell types (Treloar et al. 2010) and is attached to a basal lamina. Two subpopulations of basal cells can be distinguished, namely the self-renewing globose basal cells (GBCs) which produce olfactory sensory neurons and non-neuronal cells like sustentacular cells (SUS) (Treloar et al. 2010). Further the horizontal basal cells (HBCs), which are as well multipotent cells, which possess the capability of producing GBCs and thus constitute a supply of long-lived progenitors (Leung et al. 2007; Joiner et al. 2015).

At E10.5 - 11.5, “early neurogenesis” takes place in the OE, in which the first cohort of neurons are generated and migrate out of the OE (Beites et al. 2005). Up to day E11.5 the largest proportion of the olfactory sensory neurons are situated in a proliferative stadium (Ikeda et al. 2007; Bachmann et al. 2016).

At E12.5, the OE is composed of a pseudostratified epithelium and reveals “established neurogenesis” (Figure 3) (Beites et al. 2005; Ikeda et al. 2007).

By E13.5 the OE is organized into three divisions (apical, middle and basal), and multiplying cells have established in the apical and basal aspects (Cau et al. 2002; Ikeda et al. 2007). In the basal area, the major amount of cells are stem cells and intermediate progenitors, as that precursors of olfactory receptor neurons (Menini 2010; Gokoffski et al. 2011; Kam et al. 2014; Suzuki and Osumi 2015; Bachmann et al. 2016).

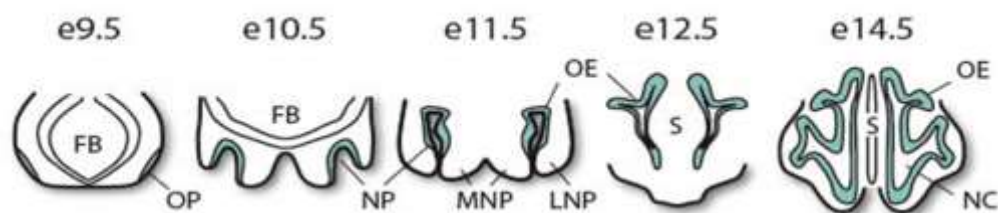


Figure 2: Schematic development of the olfactory epithelium

At E9.5 the olfactory placode starts to thicken; by E10.5 it starts to invaginate to form the olfactory pit, the onset of the nasal cavity. By E11.5 the olfactory pit has developed to a nasal cavity. At around E12.5 the OE reveals a pseudostratified epithelium. Until E14 the nasal cavity turns out to be more extensive. **Source: Kawauchi et al 2005; with friendly permission of Dr. Anne Calof**

They are able to renew, or to develop either to an intermediate progenitor, a sustentacular cell (Figure 3A), or to an olfactory ensheathing cell (OEC) that surrounds the olfactory nerve (Beites et al. 2005). The majority of apical multiplying cells are glial cells which are self-regenerative (Beites et al. 2005; Ikeda et al. 2007; Gokoffski et al. 2011; Inagi et al. 2015; Bachmann et al. 2016). The sustentacular cells exhibit a glial-like character and occupy similar functions (Farbman 1992; Ikeda et al. 2007).

Accordingly, they act in a neuroprotective manner by expressing detoxification enzymes (Ding and Coon 1988), provide structural support for ORNs (Nomura et al. 2004) and electrically segregate the cells from each other (Farbman 1992). SUS cells permeate the whole extent of the OE.

During development, the olfactory receptor neurons (ORNs) have differentiated from basal progenitors and are located in the intermediate cell compartment in between the basal and apical layer (Beites et al. 2005). Mature ORNs possess one dendrite, which is directed towards the apical side of the OE with about 12 adjacent cilia (Schwob 2002; Ikeda et al. 2007), to receive the sensory input (Figure 3).

Getting sensory input by odorants, the axonal branches of mature ORNs project directly towards the OB via the olfactory nerve layer (ONL) (Komiya and Luo 2006; Suzuki and Osumi 2015; Bachmann et al. 2016; Huilgol and Tole 2016). The axonal branches are escorted by a heterogeneous population of migratory cells, which are olfactory marker protein (OMP)- positive cells. Mature ORNs are exceptionally, due to the fact, that they are lifelong renewed during physiological turnover (Graziadei and Graziadei 1979; Schwob 2002; Suzuki and Osumi 2015). Thus, the olfactory epithelium of mice serves as a useful role model to reconstruct how neurogenesis is governed at cellular and molecular levels (Kawauchi 2005) and by which means neurons attain different destinies and regulate their correspondence amongst objectives (Kam et al. 2014; Suzuki and Osumi 2015). Due to the good bioaccessibility to ORNs and their already mentioned lifelong self-renewing potency, they constitute objects of interest for accelerated investigations with a potential of therapeutic benefit (Schwob et al. 2017).

Many transcriptional factors (TFs) playing an important role in embryonic and adult OE neurogenesis have already been identified in earlier studies (Beites et al. 2005; Treloar et al. 2010; Suzuki and Osumi 2015; Bachmann et al. 2016).

A particular cluster of these transcription factors guides the cell differentiation and specification from olfactory neuronal stem cells.

Numerous studies in vitro and in vivo have depicted exactly four levels of development in the neuronal pathway of the OE (Kawauchi 2005).

In the first developmental step, neural stem cells exhibit the transcription factors Sox2, Pax6 and Nestin (Figure 3A) (Donner et al. 2007). The induction of the olfactory placode is controlled by Pax6, Sox2 and Oct-1 (Collinson et al. 2003; Donner et al. 2007; Kam et al. 2014). Subsequently, the Mash1 and Ngn1 expression is connected with neural progenitors (Cau et al. 2002) and immature olfactory receptor neurons are expressing Tuj, HuCD, GAP43 as well as Lhx2, NCAM and Ctip2 (Arlotta et al. 2008; Enomoto et al. 2011). Finally, mature ORNs are Ctip2, OMP and NCAM positive (Figure 3A). Migratory cells from the olfactory placode which establish the olfactory mass in cooperation with ORNs axons are marked by the gonadotropin-releasing hormone (GnRH), olfactory marker protein (Treloar et al. 2010) and acetylcholine esterase (Suzuki and Osumi 2015).

BAF complexes have been identified to regulate the expression of the above mentioned transcription factors to control gene expression in a cell lineage dependent manner (Ronan et al. 2013). However, it is still unsolved and in the focus of my investigations how these TFs interact and, together with chromatin remodeling factors, coordinate OE neurogenesis (Bachmann et al. 2016). The differentiation of stem cells from pluripotent to more distinct stages of development coheres with epigenetic changes at the level of chromatin structures. The function of the BAF (mSWI/SNF) chromatin remodeling complexes will be illustrated in the following section.

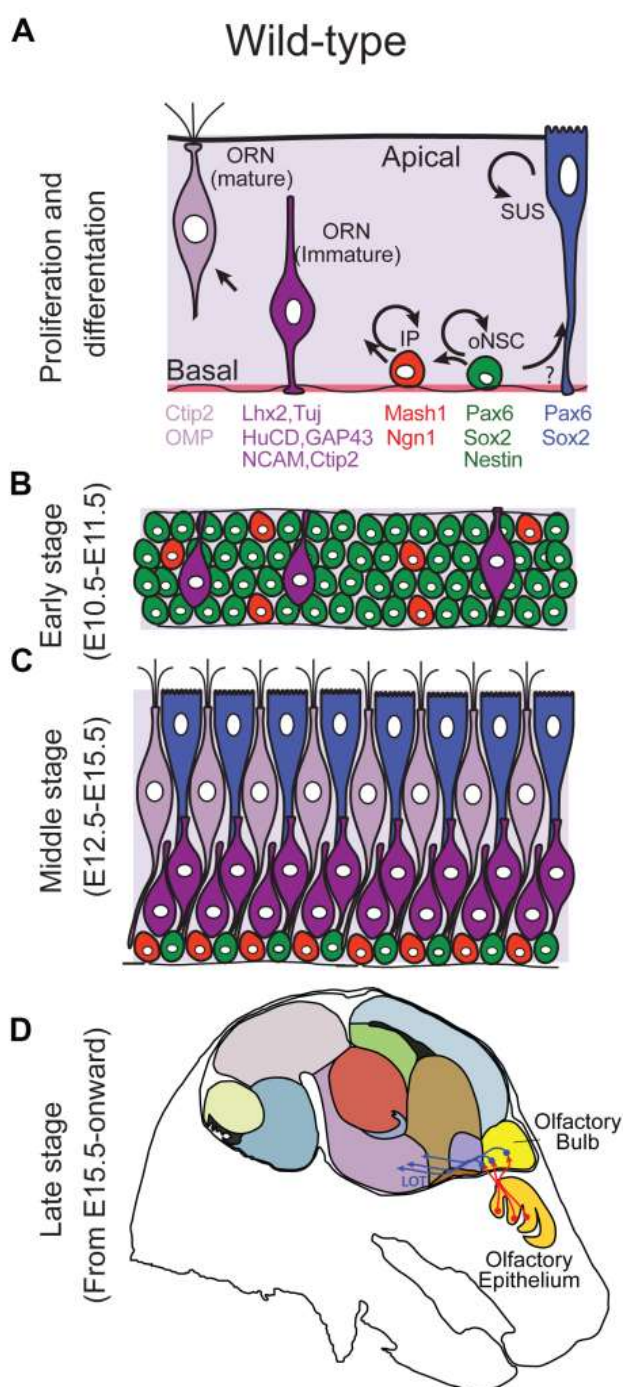


Figure 3: Schematic depiction of oNSC development and organization of the pseudostratified olfactory epithelium and axonal projections in wild-type animals

A The different cell types residing in the olfactory epithelium and the corresponding marker proteins.

B At early neurogenesis the major number of cells are stem cells, accompanied by few intermediate progenitor cells and immature neurons. During early neurogenesis there are no SUS to be found.

C In the middle stage from E12.5 – E15.5 cells are arranged in a pseudostratified manner.

D In late stages of olfactory system development, axonal projections between OE and OB, respectively olfactory cortex have been formed.

1.2 mSWI/SNF (BAF) complexes

In mammalian cells, the interchangeable Brm (Brahma) and Brg (Brahma-related gene) ATPases together with 15 other subunits form BAF (Brahma-associated factor) or mSWI/SNF complexes (Lessard and Crabtree 2010; Ronan et al. 2013).

It is known, that the ATPase subunits Brg1/Brahma, are indispensable for chromatin remodeling (Phelan et al. 1999). They occupy the role of the catalytic subunits of the complex (DelBove et al. 2011).

Mammalian BAF complexes incorporate numerous subunit combinations, being generated for instance by the position of the ATPase. By means of combinatorial assembly, hundreds of different complexes are assumed to be formed and studies have shown unique gatherings and biological particularities in different tissues.

According to the composition of subunits, the BAF complex participates in NSC supply and neuronal graduation of cells in the central nervous system (Lessard et al. 2007; Tuoc et al. 2013b; Narayanan et al. 2015a; Bachmann et al. 2016).

The universally expressed multiple-unit-complex comprises accordingly Brg1/Brm, SnF5/INI1/BAF47, BAF155, BAF170 as scaffolding subunits, as well as adjustable associated members, which relate to the developmental phase and cell type.

Most of them are essential for mouse development, as homozygous knockout mice of many BAF subunits exhibit embryonic lethality, due to developmental arrests at pre- to post-implantation transition and reveal an insufficiency of formation of pluripotent cells (Bultman et al. 2000; Kim et al. 2001; Lessard and Crabtree 2010; DelBove et al. 2011; Bachmann et al. 2016; Panamarova et al. 2016). This phenotype, however, seems to be unique for pluripotent cells, as Brg for example is not vital to the proliferation of fibroblasts or glia in mammalian embryos (Wu et al. 2007).

The underlying reason for the developmental restraints at these early embryonic phases has not been sorted out to date (Panamarova et al. 2016).

BAF complexes in general may serve as either a transcriptional activator, or repressor and can actually switch this function at the same gene (Ho and Crabtree 2010).

They enhance nucleosome replacement amongst chromosomal samples and increase the receptiveness of DNA to sequence-specific transcription factors (Lessard et al. 2007).

Furthermore, in mammalian cells, the BAF complex cooperates with many co-factors such as cell cycle control proteins including pRB, BRCA1, p21, p16, cyclin E and A (DeBove et al. 2011). This property enables the BAF complex to act as a tumor suppressor. Almost ten percent of human cancer cell lines reveal mutations or erasure of the basic subunits Brg1 and Brm (DeBove et al. 2011). A further core component, SNF5/BAF47/INI1, is an approved tumor suppressor gene, which is erased in nearly all malignant rhabdoid tumors. Thus, in tumor diagnostic, the absence of this protein serves as the diagnostic marker (DeBove et al. 2011).

As already mentioned, former studies have proved evidence that there are special compositions of subunits in each cell-type to be found. Embryonic stem cells (ESCs) for instance, are outlined by a particular embryonic stem cell (esBAF) complex with several subunits, incorporating Brg1, BAF155 and BAF250a subunits. Further the neuronal progenitor (npBAF) complex which is specific for neural progenitor cells incorporates amongst others BAF45a and BAF53a and is crucial for the regenerative and proliferative abilities of these cells (Lessard et al. 2007; Narayanan and Tuoc 2014). Contrarily, post-mitotic neurons fail to incorporate BAF45a/d, BAF53a and SS18, however generate the paralogues BAF45b/c, BAF53b and SS18l1 subunits that impart neuronal properties. Accordingly each special cell type, from neural stem cell to oligodendrocyte has its special arranged composition of subunit, cohesive with its functional role (Lessard et al. 2007; Ho et al. 2009; Kadoch et al. 2013; Narayanan and Tuoc 2014).

For a long period of time, chromatin remodeling by the BAF complex was considered to be merely a permissive instrument, crucial for gene transcription.

Yet, the BAF complex has turned out to bear an important instructional part in gene expression in several cell lineages by means of its combinative cluster and synergy with tissue-specific transcription factors (Panamarova et al. 2016).

To give an example, in embryonic stem cells (ESCs), the esBAF complex takes control over the promoters of almost every single gene in the pluripotency system and furthermore firsthand cooperates with OCT4 and SOX2, to advance the

transcription of genes which are interrelated to proliferation and renewal (Lessard et al. 2007; Panamarova et al. 2016).

However, the particular compound and structure of BAF-complexes and the connection amongst BAF-conciliated chromatin remodeling and the transcriptional program of neurogenesis in the OE is not yet solved.

1.2.1 BAF155

BAF155 is also known as SRG3 in mouse, MOIRA in *Drosophila*, and SWI3 in yeast (Kim et al. 2001; DelBove et al. 2011). Its expression pattern is ubiquitous, as also known from other core subunits (DelBove et al. 2011). However, former studies have unveiled, that in divers tissues and cell tribes, BAF155 is present in dividing stem cells and precursors, but rather decreased in terms of differentiation (Nguyen et al. 2016).

Amongst the subunits Brg and BAF47, BAF155 intensely adjusts the survival of the inner cell mass of mammalian embryos, implying an important part of BAF complexes with regard to the development of pluripotent embryonic stem (ES) cells (Lessard et al. 2007; Wu et al. 2007).

Previous studies demonstrated that an intense competition between BAF155 and BAF170 exists during embryogenesis and corticogenesis (Tuoc et al. 2013a; Tuoc et al. 2013b; Narayanan et al. 2015b). A loss of BAF170 accordingly led to the integration of supplementary BAF155 subunits, which resulted in a promoted euchromatin state and an increased binding-activity of Pax6 to its target intermediate progenitor cells, finally winding up in an enlarged cortex (Tuoc et al. 2013a; Tuoc et al. 2013b; Narayanan et al. 2015b).

The functional influence of BAF155 in maturation becomes obvious by considering prior studies with mice that reveal antagonistic responses to its expression levels: Decreased levels of BAF155 terminate in the upregulated expression of the pluripotency marker Nanog, whereas upregulated BAF155 leads to a boost of differentiation marker genes (Panamarova et al. 2016).

The gene is located on Chromosome 3p21.31- a tumor suppressor cluster region and contains 1,105 amino acids as well as diverse domains (DelBove et al. 2011). To be named are the SANT, SWIRM and leucine zipper domains (Aasland et al. 1996; Anastas and Moon 2013). Each of its protein domains has specific interactional tasks within the complex.

The SANT domain for instance, has been proven to be elementary for BRG1 linking. Moreover, the SWIRM domain of BAF155 associates with other integral parts of the mSWI/SNF complex, namely BAF60a and SNF5. It thus possesses an important role in the wake of stabilization of BAF complexes (DelBove et al. 2011; Anastas and Moon 2013).

BAF155 plays vital roles in the development of mammalian cells, as it is indispensable for transcriptional processes, proliferation and differentiation (Phelan et al. 1999; Anastas and Moon 2013; Bachmann et al. 2016).

This core subunit for instance has proved to play a significant role in preventing growth of carcinoma cells via its leucine zipper or proline/glutamine rich domain. Accordingly, the ovarian and colorectal cell lines SKOV3 and SNUC23 are lacking BAF155 protein. Hence, it reveals tumor suppressive qualities with regard to cell cycle control (DelBove et al. 2011).

1.3 Aim of investigations

The effective control of gene expression is acknowledged for being indispensable to the development of a totipotent zygote into an embryo with designated cell lineages.

Further, the accessibility of liable genes to the transcriptional machinery is proven to be dependent on chromatin remodeling complexes, such as the SWI/SNF (BAF) complex (Panamarova et al. 2016).

About the function of BAF complexes in cortical neurogenesis of the central nervous system it is already reported, that main subunits have crucial and dosage-conditioned roles in neural differentiation (Bultman et al. 2000; Kim et al. 2001a; Lessard et al. 2007; Tuoc et al. 2013b; Narayanan and Tuoc 2014; Bachmann et al. 2016) .

Nevertheless, the purpose of the BAF complex in early mouse development is still under investigation (Panamarova et al. 2016).

Hence, it remains to be clarified if BAF complexes are necessary for neural proliferation and unfolding of cells in the olfactory system and in which way they cooperate with the specific transcriptional program that controls neurogenesis in the OE (Bachmann et al. 2016). BAF155 is a core subunit of onscBAF (olfactory neuronal stem cell) and as well of ornBAF (olfactory receptor neuron) complexes and for this reason constitutes our subunit of interest in this study.

Consequently, the investigation of the influence of the knockout of the scaffolding subunit BAF155 on neuronal proliferation, maturation and differentiation of cells in the tissue of the olfactory epithelium of mice should be declared as the central issue of my dissertation.

Accordingly, the aim of this study is to depict the phenotype of BAF155cKO mice by immunohistochemistry and to draw ensuing conclusions for developmental consequences on the neural and non-neural cell-lineages of the olfactory epithelium of mice.

In order to put it into concrete terms, it remains to be determined to which extend the pool of oNSCs and IPs is impacted by a loss of BAF155 in the different developmental stages.

Moreover, it is my aim to shed light on the issue if BAF155 is like Brg not vital to the development of non-neuronal cell types (Wu et al. 2007).

I want to clarify if the maturation of oNSCs to ORNs is disturbed and sequentially assess the axonal connection between the primary and secondary olfactory pathway.

2 Material and methods

In order to analyze BAF155 expression in mutants and its meaning in the course of development of the OE in vivo, we used the Cre-loxP recombination system.

Accordingly, we interbred mice featuring floxed alleles of BAF155 (BAF155^{fl/fl}) (Choi et al. 2012; Bachmann et al. 2016) at different embryonic stages with mice expressing FOXG1-Cre, producing BAF155^{cKO}_FoxG1-Cre mutants (Bachmann et al. 2016). The animals have been treated in conformity with the German Animal Protection law and with the permission of the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES) (approval number: AZ/14/1636).

In the FoxG1-Cre mouse line, Cre recombinase is active in several areas of the head, namely the forebrain, eyes, and OE (Kawauchi 2005; Bachmann et al. 2016).

From E8.5 on, Cre activity has been detected in the anterior neural ridge, olfactory placodes and OE (Kawauchi 2005; Bachmann et al. 2016). As that, the FoxG1-Cre line is suitable for Cre recombinase activity in the initial evolution of OP/OE, when the distinction from cephalic ectoderm takes place (Hebert and McConnell 2000; Kawauchi 2005; Choi et al. 2012; Bachmann et al. 2016).

Cre is a category of recombinases which activates the splitting and recombination of genes in between two loxP detection sequences.

In our case BAF155 is floxed with two aligned loxP sequences. This mouse is crossed with an animal that inherits the FoxG1-Cre.

FoxG1 is the promotor and decides, in which cells Cre is active and thus BAF155 is deleted. Cre cuts out the floxed, looped DNA segment (Figure 4).

In order to check if the Cre mediated recombination worked out well, we applied anti-BAF155 antibody on OE sections at E10.5 to E15.5 (Figure 4). The outcome displayed an entire loss of BAF155 in the mutant embryos, which proves the knockout of BAF155.

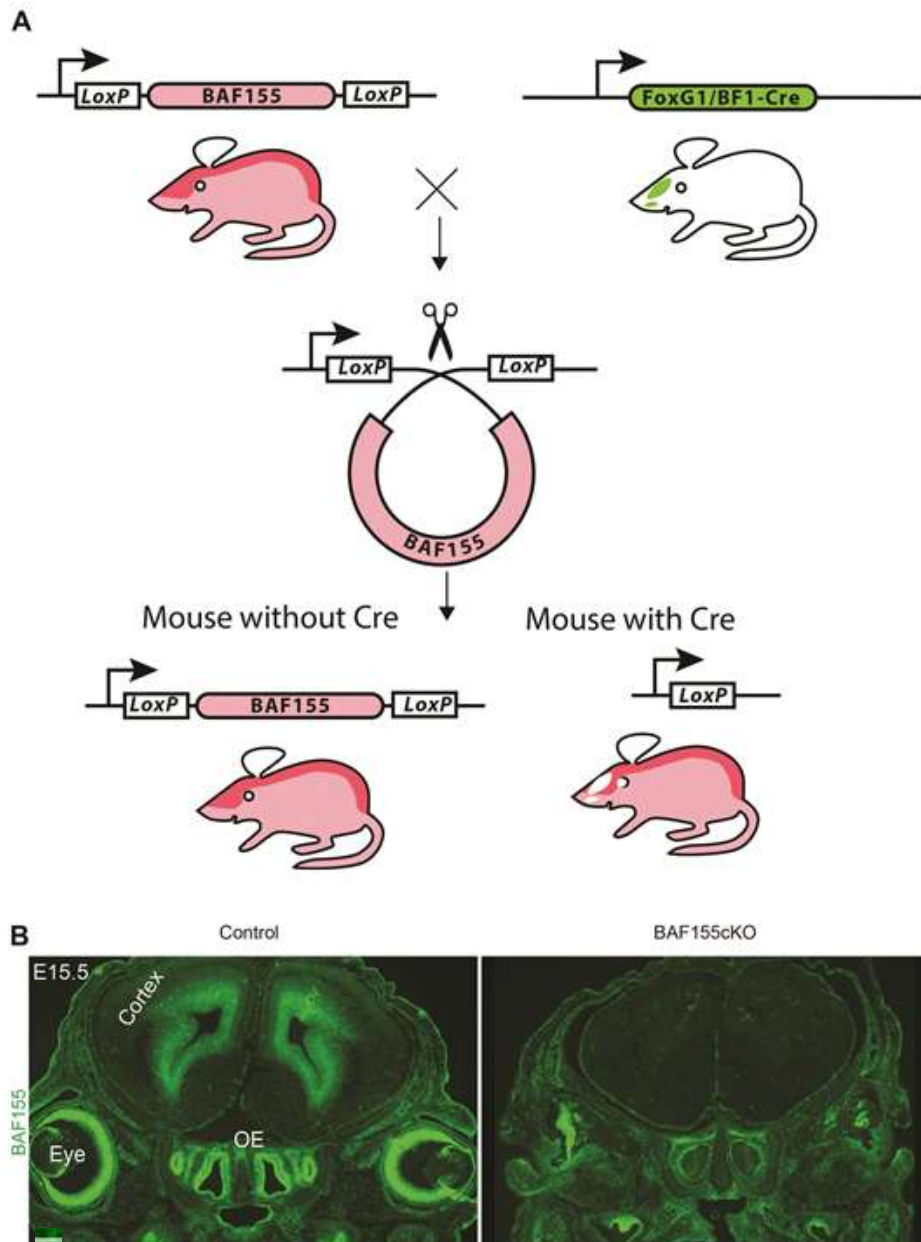


Figure 4: BAF155 cKO-mice produced by the Cre-loxP recombination system

A Cre catalyzes the splitting and cutting out of BAF155 in between the two loxP detection sequences. The areas where Cre is active, namely telencephalon, eyes, and OE are lacking BAF155.

B Immunostained sagittal head sections of control and BAF155cKO mice with BAF155 at E15.5 show an entire loss of BAF155 in the mutant in eyes, OE and cortex. Scale bar = 150 μ m.

Heterozygous mice (BAF155^{fl/+}_FoxG1-Cre) have been taken as controls. The mutant animals (BAF155cKO) pass away swiftly after birth (Bachmann et al. 2016).

BAF155 -expressing cell types were analyzed in coronal sections of the OE at E10.5, E11.5, E12.5, E13.5, E15.5 and E 18.5 by means of double label immunofluorescence microscopy, utilizing an antibody for BAF155 together with antibodies against the subsequent marker proteins: Sox2, Ki67, Nestin and Pax6 for NSCs, Mash1 for intermediate progenitor cells (IPs) (Cau et al. 2002), HuCD for immature/post mitotic neurons.

Further Sox2 and Pax6 for glial-like sustentacular cells in apical layers, Otx2, K18 and Reep6 as well for glia cells. The marker PHH3 was applied for progenitor cells at the M-phase of the cell cycle and Tuj, Ctip2, GAP43 and HuCD for post mitotic neurons, OMP and NCAM for mature ORNs (Treloar et al. 2010). Caspase3 was used as a marker for apoptosis. DAPI was applied as a marker for DNA in nuclei.

2.1 Antibodies

The following polyclonal (pAb) and monoclonal (mAb) antibodies (ABs) were utilized in this clinical trial (working dilution; sources): Pax6 mAb (1:200; DSHB), BAF155 mouse mAb (Santa Cruz), Brg1 rabbit pAb (Santa Cruz), Brg1 mouse mAb (Santa Cruz) Tuj mAb (1:200; Chemicon), Sox2 mouse mAb (R&D Systems), Ctip2 rat pAb (1:200; Abcam), Mash1 mouse mAb (1:100; BD Biosciences), HuCD mouse mAb (1:50; Invitrogen), phospho-H3 mAb (1:50; Cell Signaling), Ki67 rabbit pAb (1:50; Vector Laboratories) Casp3 rabbit pAb (1:100; Cell Signaling), OMP Goat pAb (Wako), Nestin mouse mAb (BD), GAP-43 rabbit pAb (Santa Cruz), N-CAM mouse mAb (Chemicon), Reep6 (Proteintech Group, Cat. 12088-1-AP, dilution. 1:100), K18 (1:200; Abcam; Cat. Ab52948), Otx2 (1:200; Abcam, Cat. Ab21990), Luciferase goat pAb (Acris Antibodies), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10000; Covance), HRP-conjugated goat anti-mouse IgG (1:5000; Covance), HRP-conjugated goat anti-rat IgG (1:10000; Covance), and various Alexa-conjugated second antibodies (Alexa 488, Alexa 568, Alexa 594, Alexa 647 at 1:400; Molecular Probes).

2.2 Mouse preparation and brain embedding

With two forceps, the animals head has to be separated from the body. The tail has to be collected in marked Eppendorf Save-Lock Tubes and stored by -20 °C for later genotyping. Carefully, the dura has to be removed from the brain and the brain subsequently can be lifted out and be placed in already prepared PBS (phosphate-buffered saline) on ice. The PBS+DEPC (diethyl pyrocarbonate) subsequently has to be removed and replaced by fixating PFA (paraformaldehyde) 4% for five hours.

Next, the PFA as well has to be removed and each brain is embedded in a solution of PBS+DEPC and 25% Saccharose. The brains have to be stored overnight in a -20 °C cold freezer on a tilting table.

When the brains have sunk to the bottom of the vessel, they have to be placed into a vessel with tissue-Tec® OCT TMCompound or Tissue Freezing medium and stored for 45 minutes at 4 °C. After adjusting the brains in the right position, they are definitely freezed in dry ice.

2.2.1 Cryo sectioning

The tissue samples have to be cut in slices of 10 micrometers by means of a cryostat. The cryosections have to be taken up by glass slides straight from the cryostat, marked adequately and stored in a dark freezer at -20 °C. Coronal/frontal and sagittal sections of the brains have been taken.

2.3 Immunohistochemistry (IHC)

Immunohistochemistry describes the process of visualizing an antibody-antigen interaction. It bases on the fact that antibodies are binding specifically to antigens in organic tissue. The technique contains two experimental phases.

First of all, the slide preparation as mentioned above, which is followed by different preparative measures which contain non-specific site block, primary and secondary antibody incubation, washing and counterstaining, slide mounting and storage. Second, the quantification and further analysis of the gained cell expression (Matos et al. 2010).

Specific antibodies are revealing cellular ongoings, such as proliferation or apoptosis. Proteins, for example antigens, are visualized by means of fluorescent antibodies. In order to make the antibody-antigen reaction transparent, the

antibody has to be attached to an enzyme or fluorescent dye. The primary antibodies can be classified into two groups, namely poly- and monoclonal antibodies (Matos et al. 2010). We used monoclonal and polyclonal antibodies for our investigations. Monoclonal antibodies only attach to one special epitope and are uniquely generated against the concerning antigen out of polyclonal B-cells. They are generated from hybrids and aim to more specific results (Matos et al. 2010).

Whereas polyclonal antibodies are produced by secondary immune response, after inserting animals with the special molecule, for example rabbit, goat, monkey, rat, mouse. Due to a certain inhomogeneity, polyclonal antibodies are able to bind to more than one special epitope of the same antigen. This fact leads to higher detection sensitivity (Matos et al. 2010).

For staining the tissue, we chose the indirect immunofluorescence technique. The primary AB which is applied in the first step is unlabeled and sticks to the target antigen (AG). The following fluorescent secondary AB binds with the primary AB. This second antibody has been raised versus the host species of the primary antibody (Direct vs. indirect detection in IHC | Abcam). It is of utmost importance, that the secondary AB is cultivated against the IgG of the identical species in which the primary antibody has been cultivated.

The technique of indirect immunofluorescence reveals higher sensitivity, due to higher signal amplification in contrast to direct immunofluorescence. Thus, this technique is applicable for studies of even poorly expressed antigens (Direct vs. indirect detection in IHC | Abcam).

In order to block unspecific bindings to reactive sites we used normal goat serum (Cat.S-1000, Biozol) as a buffer, right before staining the tissue. In case donkey was the host, we accordingly applied donkey serum. This course of action avoids high background staining which is able to camouflage the target antigen.

2.3.1 Procedure of immunostaining

Day 1

The formerly prepared slices have to be dried at room temperature and the tissue is circled with an ImmEdge hydrophobic pen. The samples are deposited in isotonic PBS (phosphate buffered saline) for 10 to 60 minutes, shaking under room temperature circumstances, in order to clean the cells membrane.

After cleaning, a blocking solution (5% Normal goat serum (NGS) in BPS with 0.1% Triton) for rat, mouse and rabbit primary antibodies (Abs) is pipetted on the tissue and stored for 60 minutes in a humid box.

In the next step the first primary antibody in blocking solution is pipetted on the tissue and has to be stored overnight under 4 °C.

Day 2 (in dark)

The slices have to be deposited in PBS twice for 15 minutes on a tilting table. Afterwards the first secondary antibody in blocking solution has to be applied for two hours and stored in the dark under room temperature. Subsequently, the tissue is again stored in PBS twice for thirty minutes on the tilting table and then the second primary antibody in blocking solution has to be pipetted on the tissue and be stored by 4 °C in the dark overnight.

Day 3 (in dark)

At first wash the tissue twice for 15 minutes in PBS. Then add the second secondary antibody to the blocking solution and store for two hours in a humid box under room temperature, followed by two washing-cycles of 30 minutes with PBS on a tilting table.

Subsequently, 1µl of DAPI (4',6-Diamin-2-phenylindol) in 500µl of blocking solution have to be applied on the tissue in order to stain all nuclei for later quantification.

The procedure is followed by two washing- cycles of 15 minutes in PBS. Finally, the slices have to be covered by mounting medium and a cover foil and be stored at 4 °C under dark conditions.

2.3.2 Cell counts and quantitative analysis of immunohistochemistry signal intensity

The IHC quantification was carried out with anatomically adequate coronal OE sections. DAPI⁺ (nuclear marker) cells within the OE were quantified for comparison. Cell quantifications of six fitting sections were averaged from three biological replicates (control/cKO OE pairs). The amount of marker cells of the several cell lineages was determined by utilizing the total marker positive cells alone, alternatively by normalizing the total number of DAPI-positive cells. Therefore, the equation, $\text{normalized number} = \text{marker-positive cell number} / \text{DAPI}^+ \text{ cell number}$, has proved to be suitable. To quantify the signal intensity of cytoplasm markers, fluorescent pictures of the OE were transformed to gray scale, in order to wipe out background. The occurring signal strength of pixels was scaled by the Analyze Particles function of ImageJ software and was then revealed relative to normalized values from control experiments as a percentage.

The images have been received with an Axio Imager M2 (Zeiss) combined with a Neurolucida system and confocal (Leica TCS SP5) fluorescence microscope. They have been edited with Adobe Photoshop. Contours have been arranged in each section, according to the representative occurrence of OE-specific markers. The contours were further drawn for right and left OE. The volume evaluation was performed by using Neurolucida Explorer v. 11.03. Statistical analyses are based on Student's t-test. The graphs are visualized as mean \pm SEM (standard error of mean) (Bachmann et al. 2016). All statistical tests are two-tailed, and P-values are considered to be significant for $\alpha = 0.05$.

2.3.3 Dil-labeling

The Dil-labeling experiment was performed by our collaborators in the Max Planck Institute for Biophysical Chemistry Göttingen (MPIBPC) Ahmed Mansouri and Tamara Raabe.

3 Results

3.1 Abnormal morphology of olfactory bulb and olfactory epithelium in BAF155 conditional knockout mice

As the main intention of this study, it was brought out to clarify the function of BAF155 in the neurogenesis and development of the olfactory system.

For this purpose, initially a wild-type animal and a BAF155cKO mouse at E18.5 were examined macroscopically. As shown in a dorsal view (Figure 5), the wild-type OB (indicated by white arrow) is properly formed, whereas that of the BAF155 mutant nearly failed to develop. This first result provided direction to further investigations, which focused on the changes in neurogenesis and differentiation of stem cells in the BAF155 mutant at a molecular level. Besides the missing OB and malformed OE, BAF155cKO mutants were macroscopically characterized by smaller or missing eyes and a reduction in cortical size and thickness (Figure not shown).

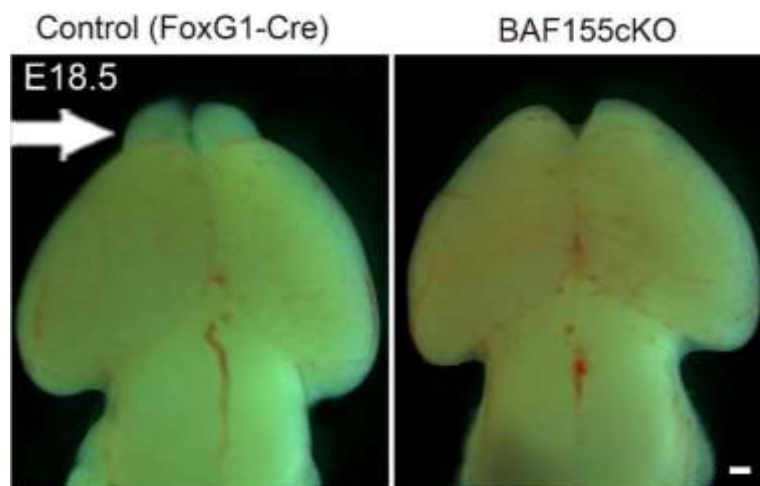


Figure 5: Macroscopic dorsal view of isolated wild-type and BAF155 mutant mouse brains at E18.5

Rudimentarily developed olfactory bulb of the BAF155cKO mouse in contrast to the wild-type FoxG1-Cre-animal (arrow) at E18.5. Scale bar = 100 μ m.

To identify the role of BAF155 in OE development, we worked with BAF155cKO_FoxG1-Cre mice. The functional reliability of Cre was immuno-histochemically confirmed in OE sections by applying anti-BAF155 antibody. The outcome was a total decline of BAF155 in the emerging OE and telencephalon of BAF155cKO mutants, which proves the efficiency of our BAF155 knock-out model (Figure 5 and 6).

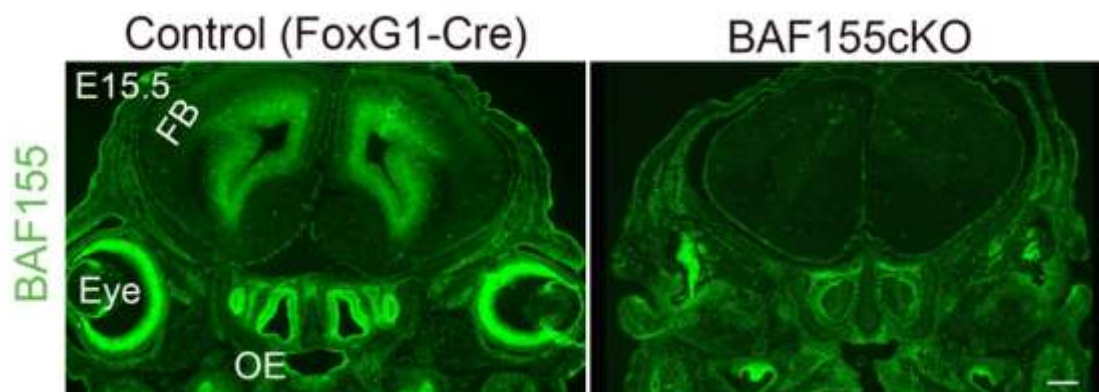


Figure 6: Expression of BAF155 in head structures of control and BAF155cKO mice at E15.5

Immunostaining of BAF155 in coronal sections of mouse heads indicates a total loss of BAF155 in developing head structures like the emerging OE, eyes and telencephalon of the BAF155cKO mutant, indicative of a successful knockout of BAF155 (FB: forebrain; OE: olfactory epithelium). Scale bar = 150 μ m.

3.2 BAF155 is expressed in the diverse cell categories of the developing olfactory epithelium of mice during early and established neurogenesis

In order to determine, whether BAF155 is expressed at the beginning of neurogenesis in the OE, IHC double-stainings for the several cell types at different embryonic stages with antibodies for BAF155 and marker proteins for cell subtypes of OE associated cells have been performed. These markers have been applied on olfactory tissue of FoxG1-cre mice (control). All cells were counter-stained with the nuclear marker DAPI.

3.2.1 BAF155 expression in early (E10.5 - E11.5) neurogenesis

I firstly concentrated on the developmental phase of early neurogenesis from E10.5 - E11.5 when NSC, intermediate progenitors (IPs) and immature neurons are mainly found (Menini 2010). The control animal in figure 7 reveals that BAF155 is present in all these important cell types at E10.5. The markers Pax6 and Sox2 for NSC are co-labeled with BAF155. This implies that these stem cells also express BAF155. We saw same results with the Mash1⁺ intermediate progenitor cells and the immature neurons marked by HuCD and Tuj. As well these cells were co-labeled with BAF155. Thus, figure 7 indicates, that BAF155 is inherent in NSC, IPs and as well in immature OSNs.

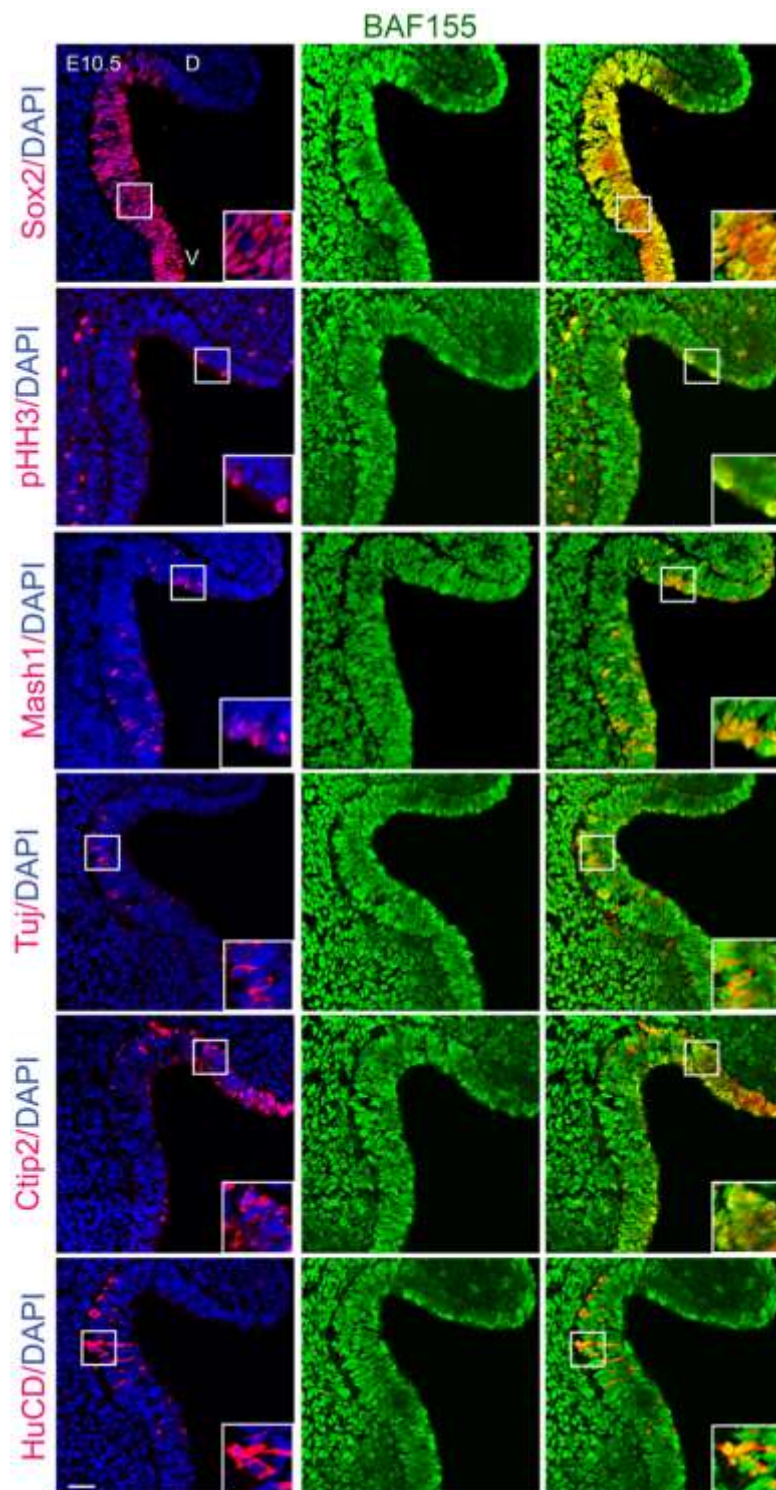


Figure 7: Double IHC-staining of olfactory epithelium from a control animal at day E10.5 in early neurogenesis

The NSC-markers Pax6 and Sox2 for NSC are co-labeled with BAF155 and appear as orange cells in the right column. This implies that these stem cells are also BAF155-expressing cells. Same results were seen with the Mash1⁺ intermediate progenitor cells and the immature neurons marked by HuCD and Tuj. BAF155 accordingly is experimentally verified in NSC, IP and immature neurons in early neurogenesis. Scale bar = 25 μ m, sagittal view.

3.2.2 BAF155 expression in established (> E12.5) neurogenesis

We investigated whether BAF155 is also expressed in the developmental stage of established neurogenesis at E12.5 in wild type mice. Pax6, Ki67 and Sox2 are marking glial cells in the apical region and stem cells in the basal area (Donner et al. 2007). All three markers are co-localized with the BAF155 staining in wild type animal. Mash1 as a marker for intermediate progenitors, which are predominantly restricted to the basal lamina (Cau et al. 2002), are as well expressing BAF155. Similar co-expression patterns were found with HuCD and Ctip2 staining. To merge these findings, we identified the expression of BAF155 in Pax6⁺, Sox2⁺ oNSCs, as well as in ki67⁺ proliferating progenitors and Mash1⁺ neuronal progenitors. The same applies to Ctip2⁺, HuCD⁺ ORNs and Sox2⁺ and Pax6⁺ oNSCs in the basal layer and proliferative neuroepithelial sustentacular cells (SUS) in the apical layers from E12.5 on (Figure 8). This result is essential and constitutes the basis for further investigations.

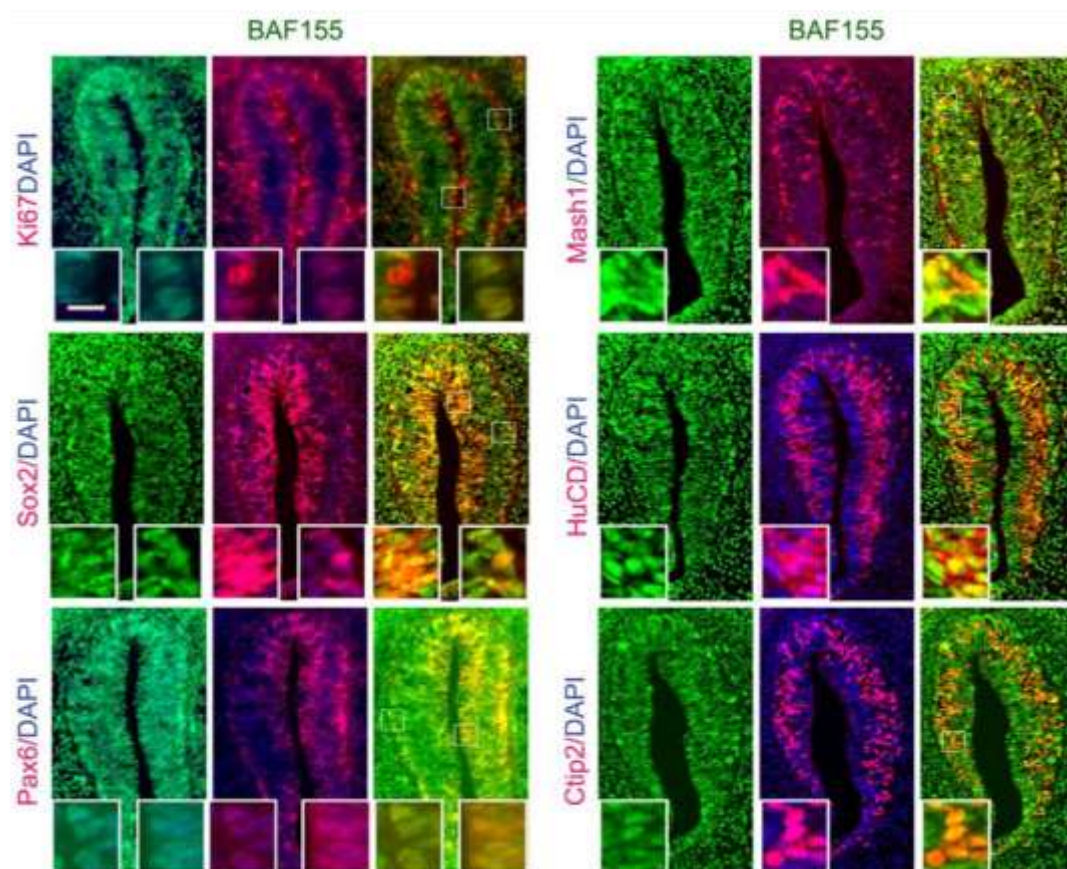


Figure 8: BAF155 expression in the olfactory epithelium during established neurogenesis at E12.5

In the right columns BAF155 is colabeled with Ki67, Sox2, Pax6 markers for stem cells in the basal area and glial cells in the apical region, these colabeled cells appear orange. Same applies to Mash1⁺ neuronal progenitors and HuCD⁺ and Ctip2⁺neurons. As well these cells are colabeled with BAF155 in the right column and as that are BAF155-expressing cells. Scale bar = 50 μ m.

3.3 Delayed transformation of olfactory placode to mature olfactory epithelium in BAF155cKO mutants

For the purpose of investigating the OE development and widening in the absence of BAF155, sections of mutant and control animals in early neurogenesis at E10.5 (Figure 9A), E11 (Figure 9B) and established neurogenesis at E13.5 (Figure 9C) and E15.5 (Figure 9D) have been stained.

3.3.1 Reduced thickening of the OP and delayed state of OE transformation during early neurogenesis in the BAF155cKO mutant (E10.5, E11.5)

Our attention was drawn to the fact, that at E10.5 the thickening of the mutants' placode is reduced in volume compared to the wild-type, and that it is not BAF-expressing (Figure 9A).

By comparing the OE of control animals and BAF155cKO mice at E11.5 in a rostral, medial and caudal section, it becomes apparent, that the control mouse reveals a proper developed OE, whereas the mutant features a delayed state of transformation, which is developmentally appropriate for E10 - E10.5 (Bachmann et al. 2016) (Figure 9B).

At E11.5 the invagination process of the OE to a complete nasal cavity should have contracted to form the nares (Menini 2010; Bachmann et al. 2016).

However, the mutant phenotype reveals a structure which still remains a cuplike morphology which is characteristic for E10.5 (Menini 2010) (Figure 9A&B).

The thickening of the mutant placode in comparison to the wild-type is clearly reduced even at E11.5. In all three consecutive slides from rostral to caudal we have similar results of delayed transformation from OP to OE in the BAF155cKO mouse.

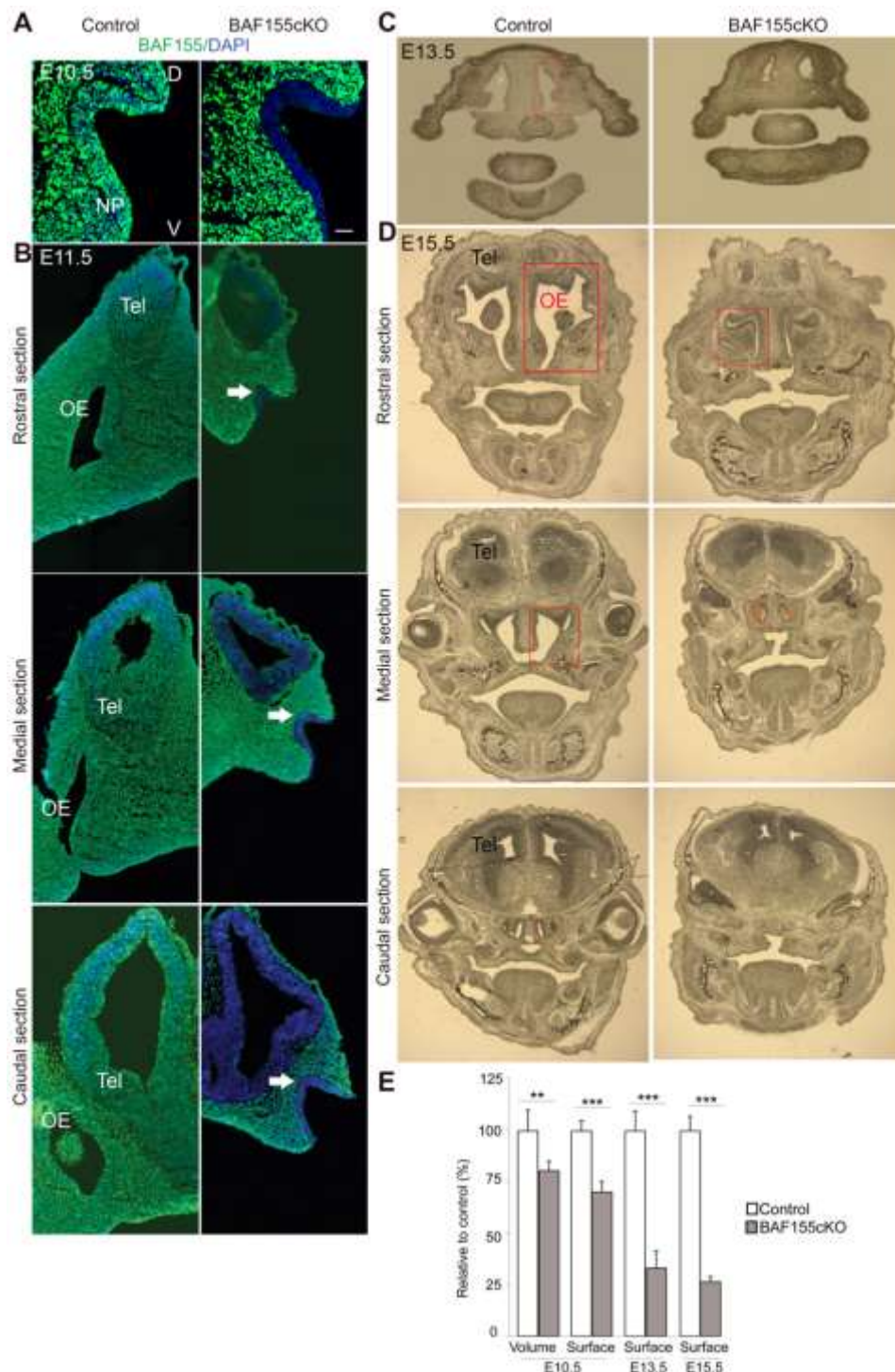


Figure 9: Chronologic examination of the control and BAF155cKO mice in the developing olfactory epithelium

A Sagittal section of mutant and control nasal pit (NP; arrow) at E10.5, stained with BAF155 and DAPI reveals missing BAF155 expression in the BAF155cKO. **B** Staining with BAF155 and DAPI at E11.5 of rostral, medial and caudal OE sections. It is visible that the control mouse reveals a properly invaginated nasal cavity, whereas the mutant OE reveals a delayed state of transformation with a cuplike morphology, which is appropriate for E10 - E10.5 (arrow). **C, D** Images reveal coronal sections of control and BAF155cKO embryos at E13.5 and E15.5. Notably thinner and lesser OE of BAF155cKO embryos in comparison to the wild-type were observed. **E** Quantification of volume and surface parameters of mutants' OE relative to control at E10.5 and surface parameters at E13.5 and E15.5 reveals a significant decline in the mutant

OE (D: dorsal; V: ventral; Tel: telencephalon). Scale bar = 25 μ m. Values are reported as means \pm SEM (*P < 0.05, ** P < 0.01, *** P < 0.001).

3.3.2 Reduced volume and surface parameters of the BAF155cKO OE in established neurogenesis (E13.5, E15.5)

At E13.5, BAF155 conditional knockout mutants exhibit a macroscopic phenotype which is reduced in volume and surface parameters, although it has a properly formed OE (Figure 9C). At E15.5 the developmental contrast between mutant and wild-type is even more striking (Figure 9D) and depicted in a rostral, a medial and a caudal view.

Figure 9E makes transparent, that the surface and volume area at E10.5 of the mutant animal relative to the control mouse is significantly smaller (Bachmann et al. 2016).

This discrepancy in surface dimension of the OE between control and BAF155 cKO is even more striking at E13.5 and E15.5 (Figure 9E).

In the rostral, medial and caudal section, the surface relative to control was barely 25%. These results give direction for an indispensable role of BAF155 in the development of the OE. It allows the assumption that histogenesis is affected by means of increased apoptosis and/or defective progenitor development (Bachmann et al. 2016).

3.4 Defective neurogenesis and altered cell differentiation in BAF155 conditional knockout mice

As the previous results indicate that the absence of BAF155 hampers OE development, I next investigated the possible mutation in proliferation and further differentiation of oNSCs.

The reduced size of the mutants' OE allows the presumption of defects in the previously named processes or the occurrence of increased apoptosis.

3.4.1 During early neurogenesis (E10.5 – E11.5)

Generally, in early neurogenesis, which includes the span of time from E10.5 to E11.5, the majority of cells are oNSCs (Pax6⁺, Sox2⁺) and several neuronal progenitors (Mash1⁺, Ki67⁺), as well as immature ORNs, marked by HuCD, Lhx2 and Tuj1.

Stem cells: Pax6⁺, Sox2⁺

At E10.5 the markers Pax6 and Sox2 for stem cells are significantly reduced relative to control in the OE of the mutant embryo (Figure 10).

Intermediate progenitor cells: Mash1⁺ and immature neurons: HuCD⁺, Tuj1⁺

Contrary to the diminished amount of stem cells, the OE neurogenesis of intermediate progenitors and immature neurons in this early developmental stage (E10.5) appeared unaffected due to the slight reduction of the progenitor specific markers Mash1 (Figure 11) and Tuj (Figure 12) and the even upregulated marker HuCD (Figure 11). Nevertheless, this data indicates BAF155 is indispensable for the normal generation of neurons early in OE development.

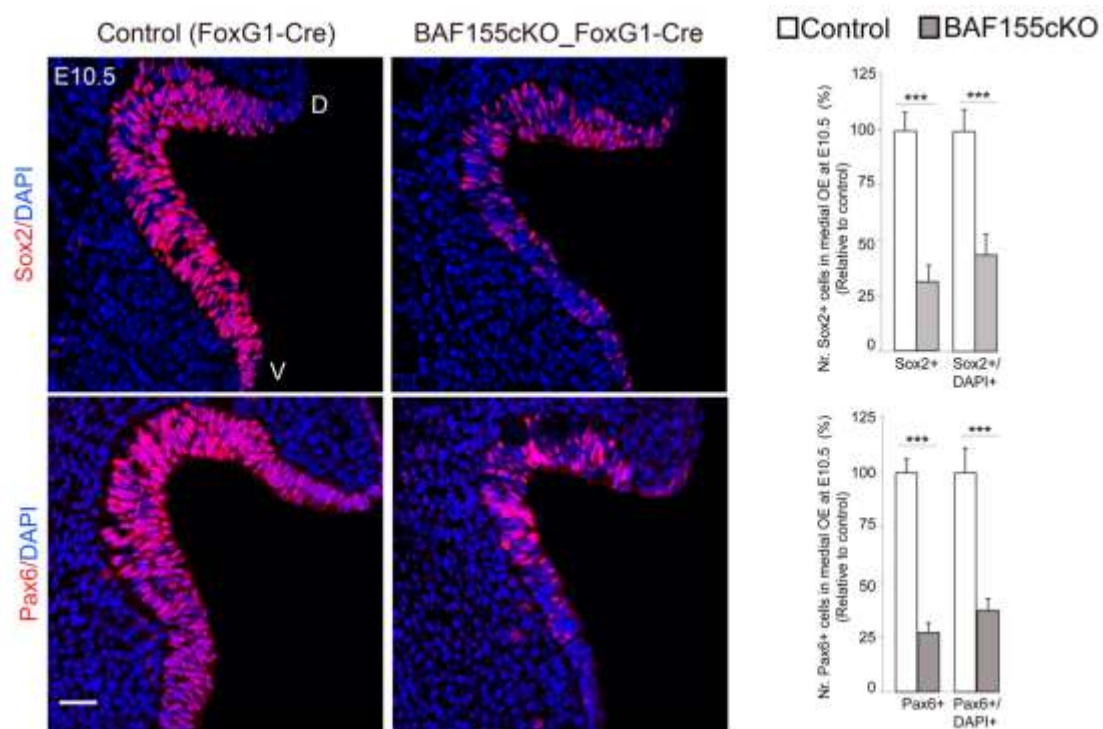


Figure 10: Reduced lot of oNSCs in embryonic BAF155cKO OE at E10.5

Images of OE sections from control and BAF155cKO animals revealing IHC confirmation of the oNSC markers Sox2 and Pax6 at E10.5. Visibly less oNSCs marked by Sox2 and even Pax6 in the BAF155cKO animal. Statistical quantification is shown in the panels. A significant reduction in both cases is to be recognized (D: dorsal; V: ventral). Scale bar = 25 μ m. Values are reported as means \pm SEM (*P < 0.05, ** P < 0.01, *** P < 0.001).

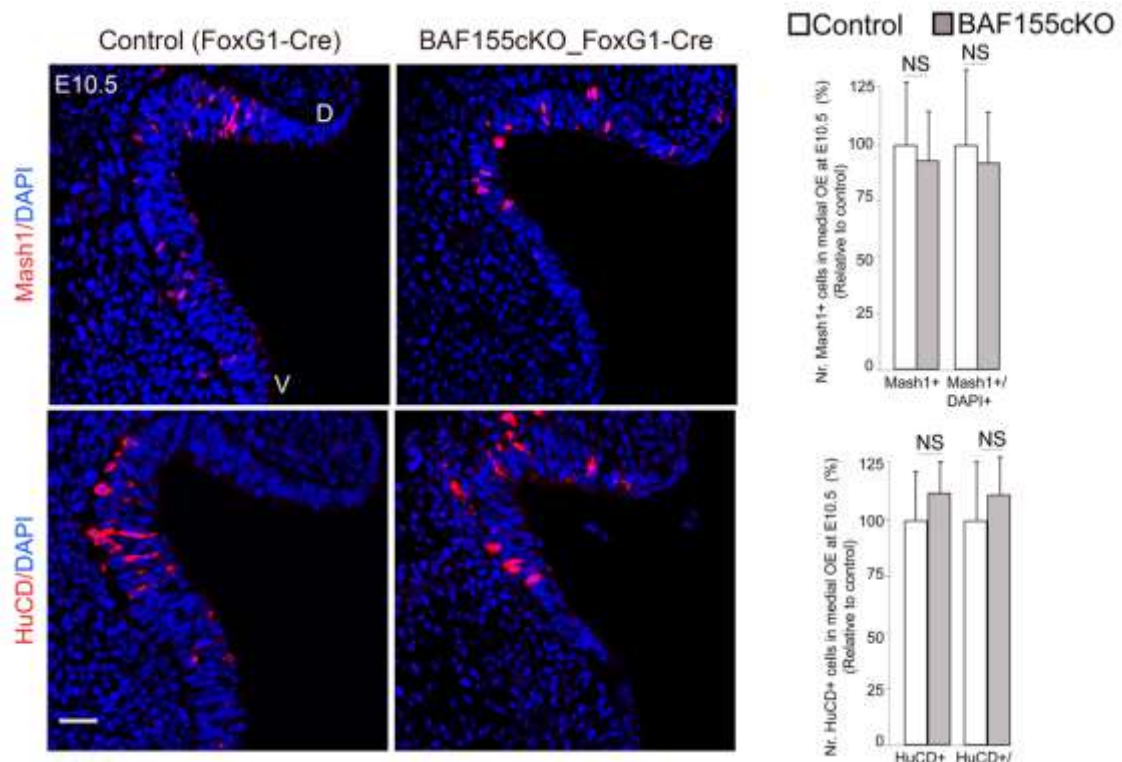


Figure 11: Early-stage neurogenesis of progenitors and immature neurons is unaffected at E10.5

Depiction of OE sections from control and BAF155cKO OE at E10.5 and quantification show that the progenitor specific Mash1⁺ cells and immature HuCD⁺ neurons are not reduced in the mutant animal (D=dorsal; V= ventral). Scale bar = 25 μ m. Values are reported as means \pm SEM, (NS: not significant).

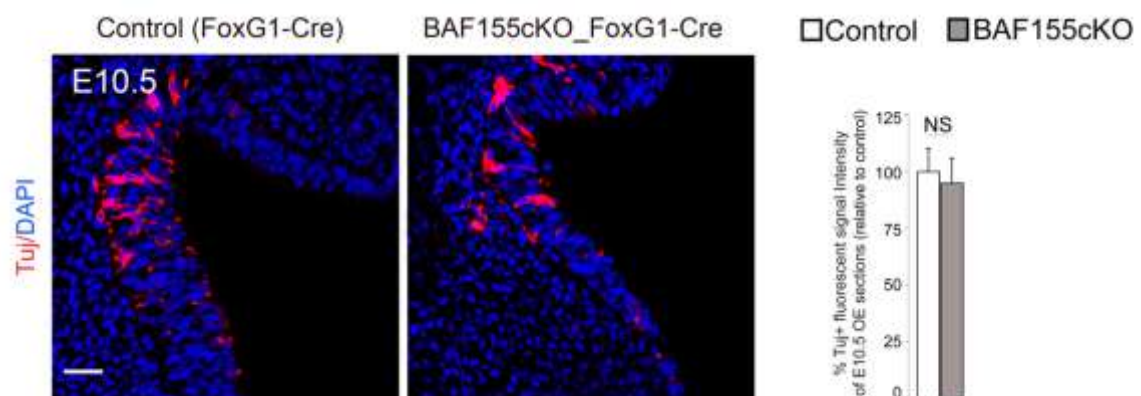


Figure 12: Early-stage neurogenesis of immature neurons is unaffected at E10.5

Depiction of OE sections from control and BAF155cKO OE at E10.5 and the quantification panel reveal that the marker Tuj for immature, post-mitotic neurons is not significantly decreased in the mutant animal at E10.5. Scale bar = 25 μ m. Values are reported as means \pm SEM, (NS: not significant).

3.4.2 During established neurogenesis (> E12.5)

The following period of established neurogenesis from E12.5 onwards is significant for proliferation and further development of precursors, namely oNSCs, which are designed to produce ORNs, the sensory perception cells of the OE. Under physiologic conditions, progenitors are outsourced into the basal epithelium around E13.5 (Kam et al. 2014; Bachmann et al. 2016). We tried to investigate, whether the self-regeneration of oNSCs and the differentiation of these cells to ORNs and SUS is impaired in late neurogenesis by a knockout of BAF155.

Stem cells: Sox2⁺, Pax6⁺, Nestin⁺ and sustentacular cells: Sox2⁺, Pax6⁺, Otx2⁺, K18⁺, Reep6⁺

At E 13.5 glial and sustentacular cells are marked apically by Sox2 and Pax6, whereas stem cells are marked basally by these markers (Figure 13). Likewise to E 10.5, this staining offers, that in the BAF155cKO animal a clear reduction of stem cells in the basal area can be demonstrated.

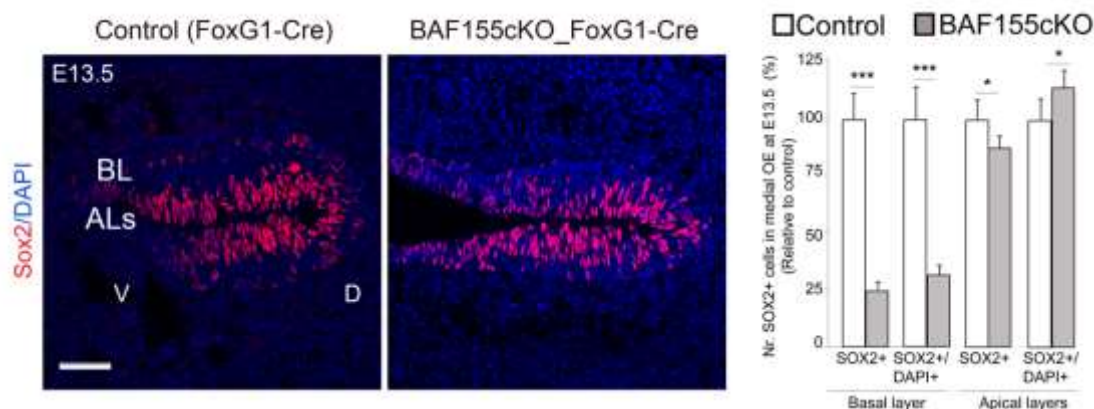


Figure 13: Depleted pool of stem cells in E13.5 OE of BAF155cKO mice and preserved pool of SUS cells

Sox2⁺ stem cells in medial OE of the mutant are clearly reduced relative to control in the basal layer. Note, that the apically located Sox2⁺ SUS cells are not downscaled (B: basal layer; ALs: apical layers; V: ventral; D: dorsal). Scale bar = 50µm. Values are reported as means ± SEM (*P < 0.05, ** P < 0.01, *** P < 0.001).

The statistic quantification of figure 13 reveals that basally located Sox2⁺ cells of the mutant are reduced by 75% relative to control at E13.5. Similar to E10.5 the Sox2⁺ stem cells are reduced in the OE of the BAF155cKO at E13.5. Strikingly,

the glial cells, marked apically by Sox2, are not decreased in number (Figure 13). That leads to the assumption that in late neurogenesis the process of gliogenesis is not affected. In order to substantiate this result, the expression pattern of further SUS markers, namely Otx2, Cytokeratin 18 (K18) and Reep6 have been visualized.

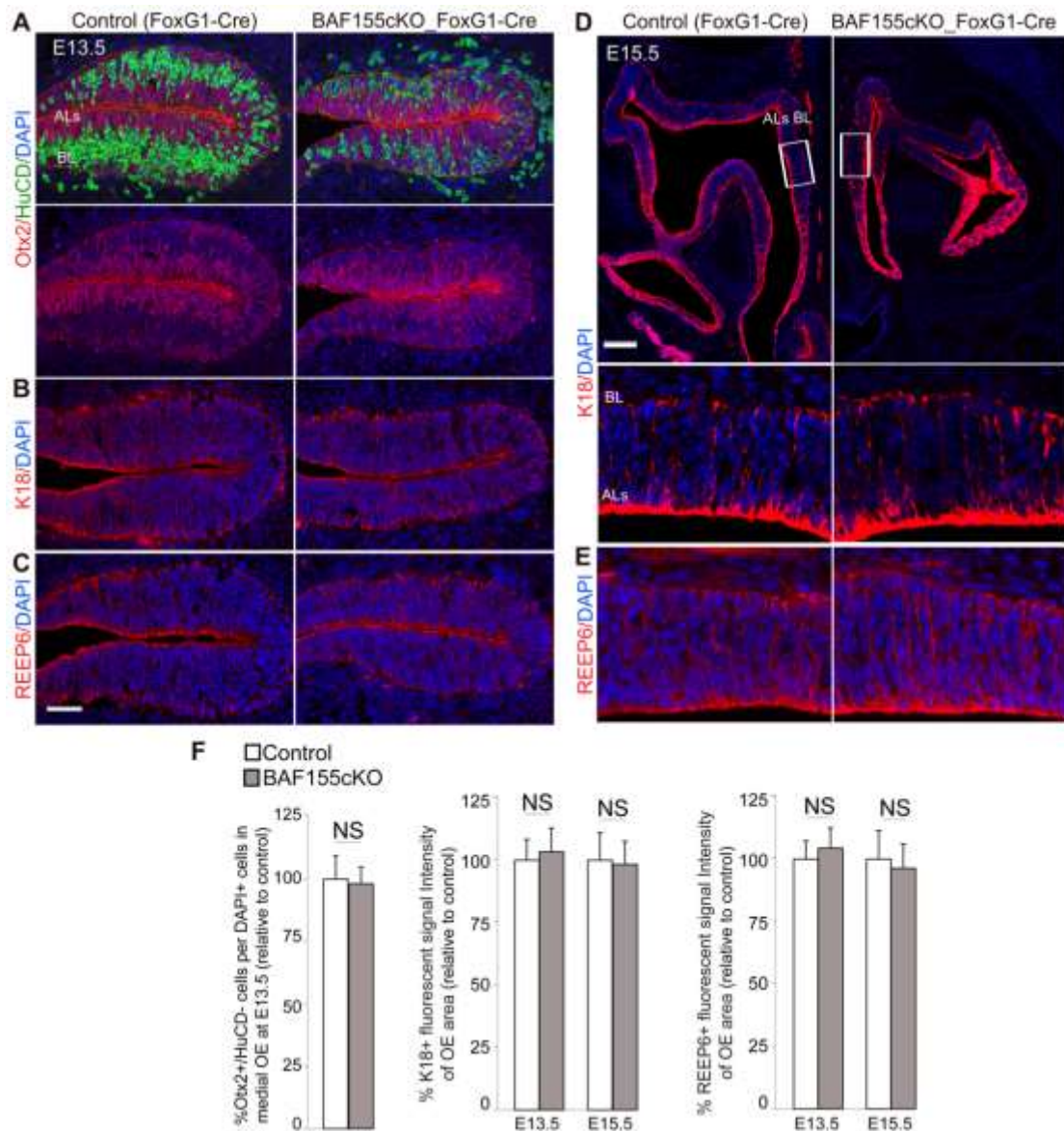


Figure 14: No reduction of glial cells in established neurogenesis of control and mutant OE

A-C Representative images of coronal sections from control and BAF155cKO embryos reveal IHC confirmation of the SUS markers Otx2, K18 and Reep6 at E13.5

D and **E** at E15.5 K18 and **E** Reep6 in the control animal as well as in the mutant. **F** Statistical quantification is illustrated in panels. Scale bars = 50µm (A, B, C) and 150 µm (D). Values are reported as means ± SEM, (NS: not significant).

Accordingly, these investigations detected an equal percentage of Otx⁺/ HucD⁻ cells in control and mutant OE at E13.5 (Figure 14A&F), and a correlative number of K18 and REEP6 positive cells among OE of control and BAF155cKO animals at E13.5 and E15.5 (Figure 14B,C,D,E,F).

To affirm the previous result of an impaired neurogenesis in BAF155cKO mice, I additionally used the marker Nestin. Even the stem cell population of the Nestin⁺ cells at E13.5 is notably reduced in the BAF155cKO animal (Figure 15).

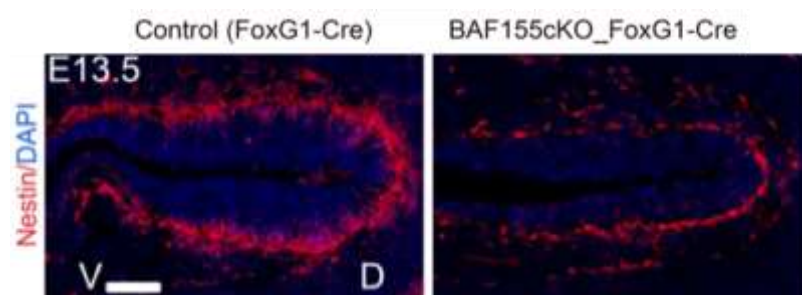


Figure 15: Decrease of Nestin⁺ stem cells at E13.5 in the mutant OE

Images of coronal sections from a control and BAF155cKO embryo at E13.5, showing IHC detection of the stem cell marker Nestin. The mutant OE on the right side reveals an obvious decrease of Nestin⁺ stem cells. Scale bar = 50 μ m.

Intermediate progenitor cells: Mash1⁺

The population of intermediate progenitor cells was visualized by using the marker Mash1. In contrast to E10.5, where the number of intermediate progenitors remained stable in the BAF155cKO OE (Figure 11), at E13.5 the number of Mash1⁺ cells decreased by 75% relative to control (Figure 16).

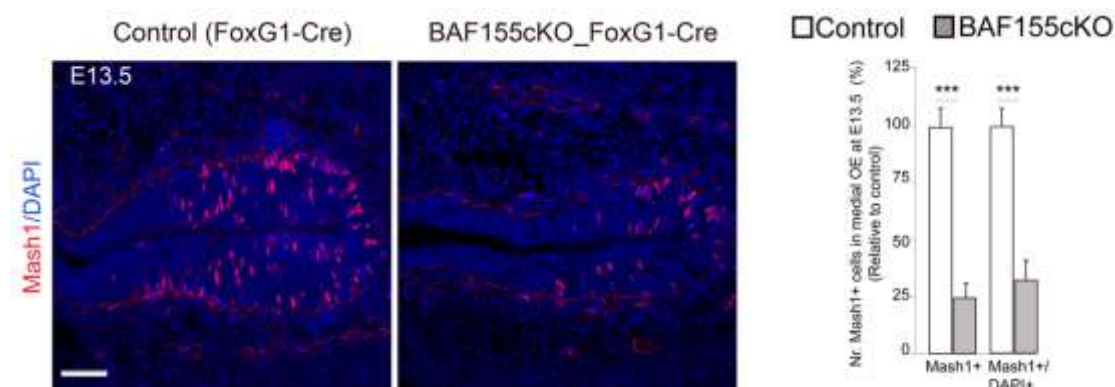


Figure 16: Decreased amount of Mash1⁺ intermediate progenitors in mutant OE at E13.5

IHC with Mash1 and statistical quantification reveals a significant reduction of progenitor cells in medial OE in mutant relatively to control. Scale bar = 50 μ m. Values are reported as means \pm SEM (*P < 0.05, ** P < 0.01, *** P < 0.001).

Immature olfactory receptor neurons: HuCD⁺, GAP43⁺, Tuj⁺

To determine whether the group of immature ORNs of the mutant embryo remains as stable as on day E10.5, IHC was performed with the markers HuCD, GAP43 and Tuj with olfactory tissue from E13.5. We thus investigated if BAF155 is indispensably needed for a physiological late neurogenesis. These immature neurons of interest are located in the middle layer of the OE. As opposed to what was found at early neurogenesis stages, the number of immature ORNs stained by HuCD is decreased at E13.5 to approximately 50 percent relative to control (Figure 17). The Tuj⁺ ORNs are 40% less in the BAF155cKO OE compared to control and the GAP43⁺ cells are 25% less than that in control (Figure 18).

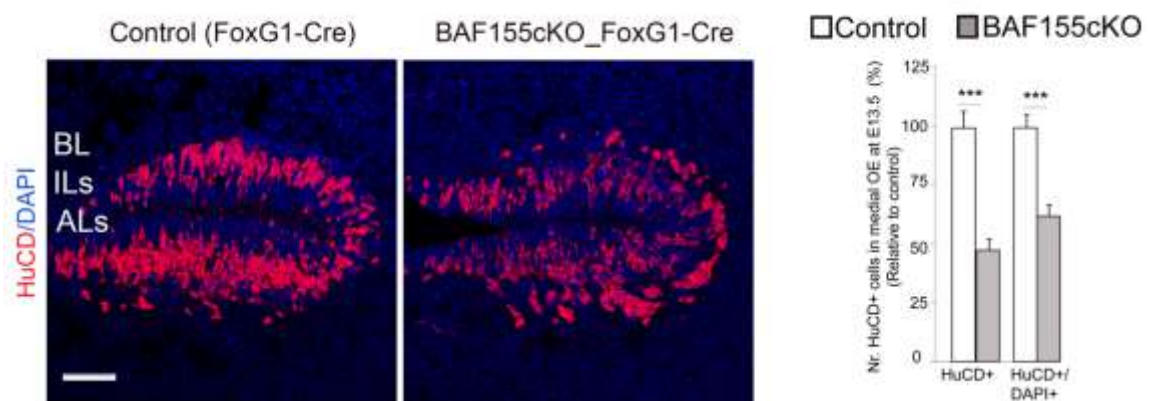


Figure 17: Reduction of HuCD⁺ immature ORNs indicative of disturbed late neurogenesis at E13.5

IHC of BAF155cKO and control OE with HuCD and statistical analysis show about 50% decrease in the number of immature ORNs (BL: basal layer; ILs: intermediate layers; ALs: apical layers) in the BAF155cKO embryo. Scale bar = 50 μ m. Values are reported as means \pm SEM (*P < 0.05, ** P < 0.01, *** P < 0.001).

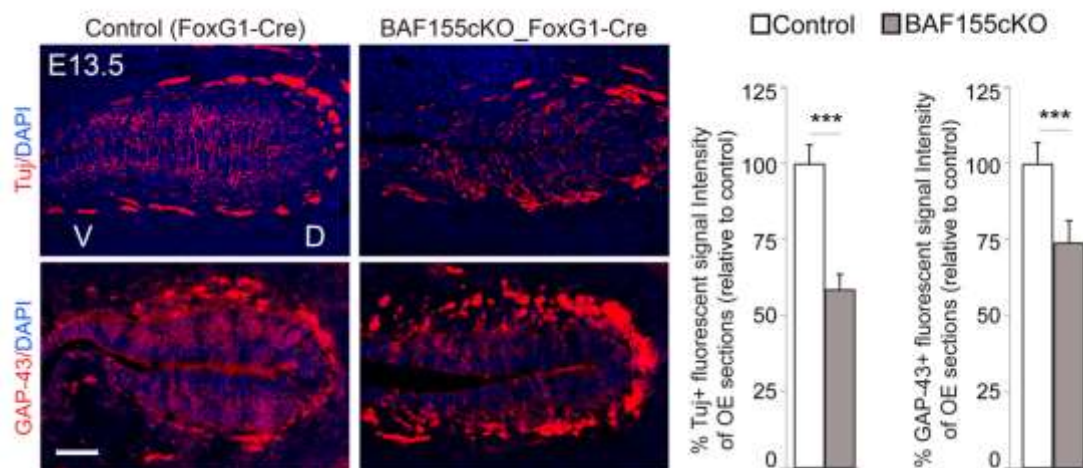


Figure 18: Reduced amount of ORNs in mutant OE at E13.5

Up to 25% less immature neurons marked by GAP43 in the BAF155cKO in contrast to those found in the control OE. Tuj⁺ cells were 40% less in the mutant at E13.5 (V: ventral; D: dorsal). Scale bar = 50 μ m. Values are reported as means \pm SEM (* P < 0.05, ** P < 0.01, *** P < 0.001).

3.5 BAF155 is vital to neuronal maturation in the olfactory epithelium of mice

The last group of cells which I focused on is that of unfolding olfactory receptor neurons. Ctip2 is a key marker for the maturation of olfactory receptor neurons (Arlotta et al. 2008; Enomoto et al. 2011) and as such it is useful to measure the amount of immature and adult ORNs. Thus, I next examined whether the deletion of BAF155 disturbs maturation in OE. For this purpose, tissue from E10.5, E11.5 and E13 has been stained.

3.5.1 Loss of Ctip2 in the course of development from immature to mature ORN in the BAF155cKO mutant

The stainings in figure 19 respectively compare a sagittal section of a control and a mutant olfactory epithelium with regard to the expression of the marker Ctip2 for immature and mature ORNs. At all three stages of neurogenesis, we notice a loss of Ctip2⁺ neurons in the BAF155cKO mutant (Figure 19B). At E10.5 and E13.5, the BAF155cKO OE exhibits a clearly decreased amount of Ctip2⁺ cells. At E11.5 Ctip2 is almost completely wiped out in the OE mutant. The fact, that in mutants the amount of differentiated OSNs is severely diminished, leads to the assumption that BAF155 mutants may have a problem with maturation.

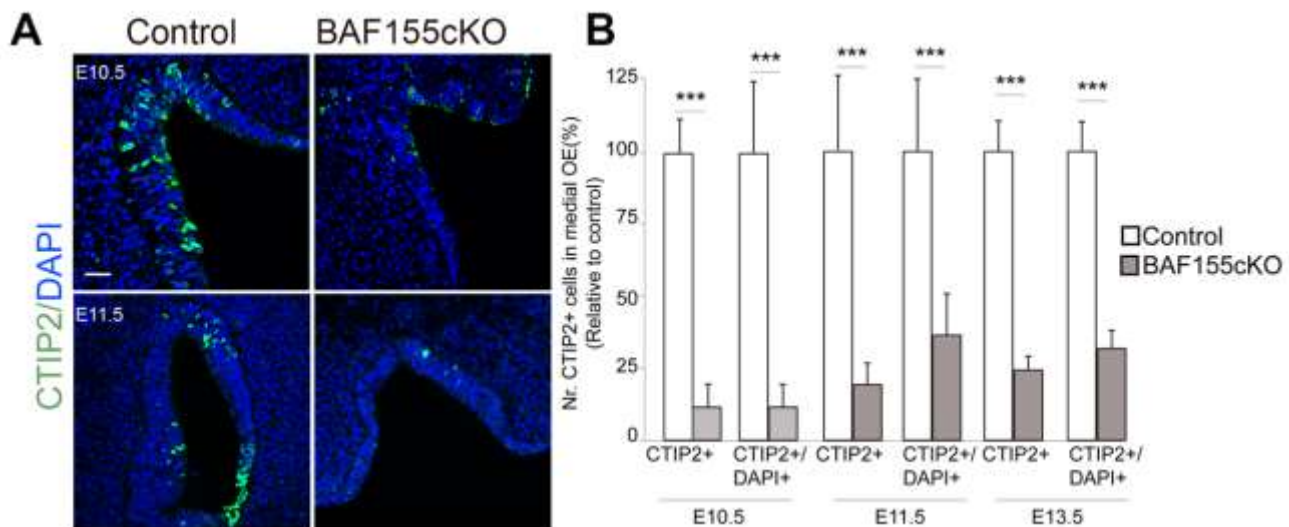


Figure 19: BAF155cKO mutants might have a defect in the maturation of ORNs

A Sagittal section of mutant and control OE at E10.5, E11.5 and E13.5 stained with Ctip2. Note that the amount of Ctip2⁺ immature and mature neurons in the BAF155cKO tissue is significantly decreased. **B** The statistical evaluation reflects this reduction of cells in the mutant OE at E10.5, E11.5 and E13.5. Scale bar = 25 μ m. Values are reported as means \pm SEM (* P < 0.05, ** P < 0.01, *** P < 0.001).

3.5.2 Loss of OMP⁺ and Tuj⁺ neurons at E13.5 and E15.5

In order to confirm the thesis of an impaired maturation in BAF155cKO mutants, the marker OMP for mature neurons at E13.5 and E15.5 has been used. The data reveals that similar to Ctip2 stained ORNs, the OMP⁺ mature ORNs are almost completely wiped out in the BAF155^{-/-} OE at E13.5 (Figure 20C) and on E15.5 (Figure 20B).

The arrow in figure 20 points on the region of the forebrain where the OSN axons reach the telencephalon in order to induce the formation of the OB (Treloar et al. 2010). These axons are accompanied by a population of OMP⁺ migratory cells and together form the so called “migratory mass” (Treloar et al. 2010). The explicit features and nature of these migratory cells is still under investigation, but the OMP⁺ ones presumably act as a kind of directory for growing OSN axons (Conzelmann et al. 2002).

Besides the decreased number of OMP⁺ olfactory receptor neurons in the BAF155cKO OE, the BAF155cKO also lacks these migratory cells in the area of the forebrain at E15.5. These results led to the implication that the knockout of BAF155 is associated with an impaired development of oNSCs to mature ORNs.

Coronal sections of the OE and rostral aspect of the forebrain of E13.5 were further analyzed by IHC applying anti-Tuj antibodies (Figure 20A).

No Tuj⁺ axonal tracts of immature neurons enter the olfactory bulb in BAF155cKO deficient mice.

In contrast, the axons of immature OSNs in the control OE presented as a fibro cellular mass (Figure 20A, arrow).

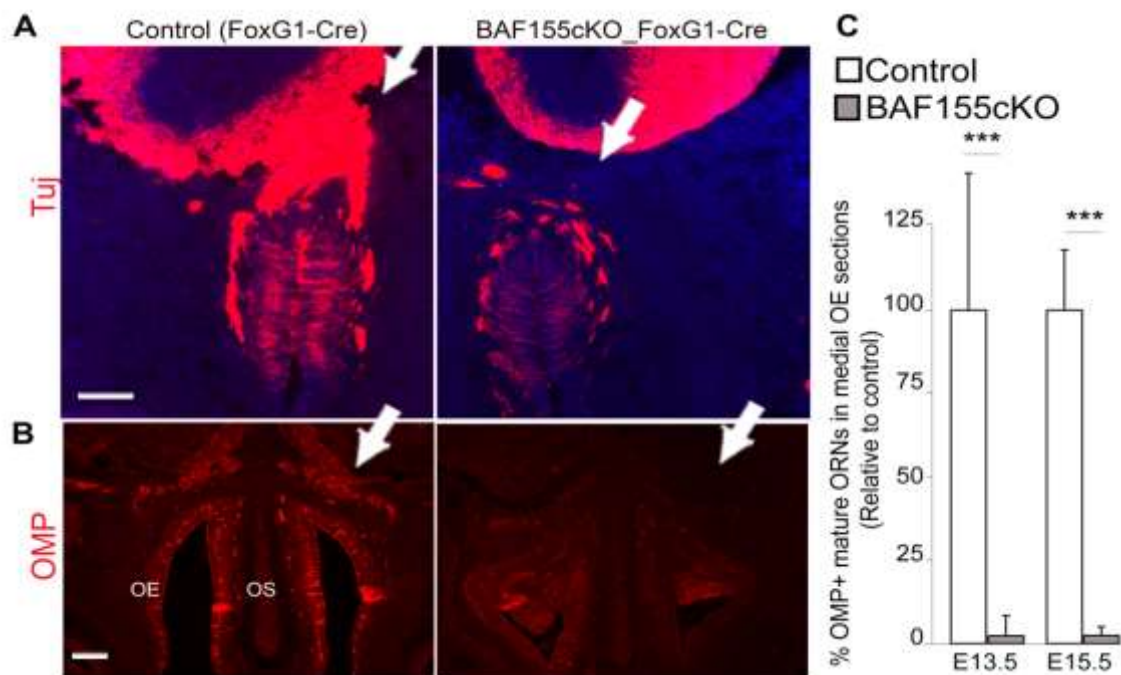


Figure 20: Loss of mature OSNs and their axonal outgrowth in BAF155cKO embryos

A & B Coronal sections of the OE and frontal telencephalon. IHC with anti-Tuj antibodies staining immature neurons at **E13.5** and anti-OMP antibodies for mature neurons at E15.5. **A** The axons of immature Tuj⁺ OSNs arrive at the OB in the control animal. Having an eye on the BAF155cKO animal the axonal outgrowth is absent (arrow). **B** Immunohistochemical stainings of coronal sections at E15.5 with an anti-OMP antibody reveal a considerably decreased amount of OMP⁺ neurons and further a loss of axonal outgrowth of ORNs to the OB (arrow). **C** Statistical evaluation of OMP⁺ mature ORNs in control OE and BAF155cKO phenotype at E13.5 and E15.5 substantiate the significant decrease of mature ORNs (OE: olfactory epithelium, OS: olfactory septum). Scale bars = 100 μ m (A) and 150 μ m (B). Values are reported as means \pm SEM (*P < 0.05, ** P < 0.01, *** P < 0.001).

3.6 Defective neurogenesis in the mutant olfactory epithelium is not due to increased apoptosis

Due to the decreased amount of oNSCs in early and established neurogenesis, the decreased number of Ctip2⁺ and OMP⁺ immature and mature neurons and as well as the reduction of immature neurons in established neurogenesis, it was to question if the disturbed neurogenesis in the BAF155cKO mutants is due to an increased cell death. For that reason, IHC analysis with anti-active-Caspase3, a marker for apoptosis at different embryonic stages has been applied on olfactory epithelium of E10.5 and E15.5 (Bachmann et al. 2016).

The data reveals that the control and as well the BAF155^{-/-} mouse only exhibit very few Casp3⁺ apoptotic cells on day E10.5 and E15.5 (Figure 21 & Figure 22). It can be concluded, that BAF155 cKO mice reveal no increased apoptotic ongoing, as reviewed by IHC.

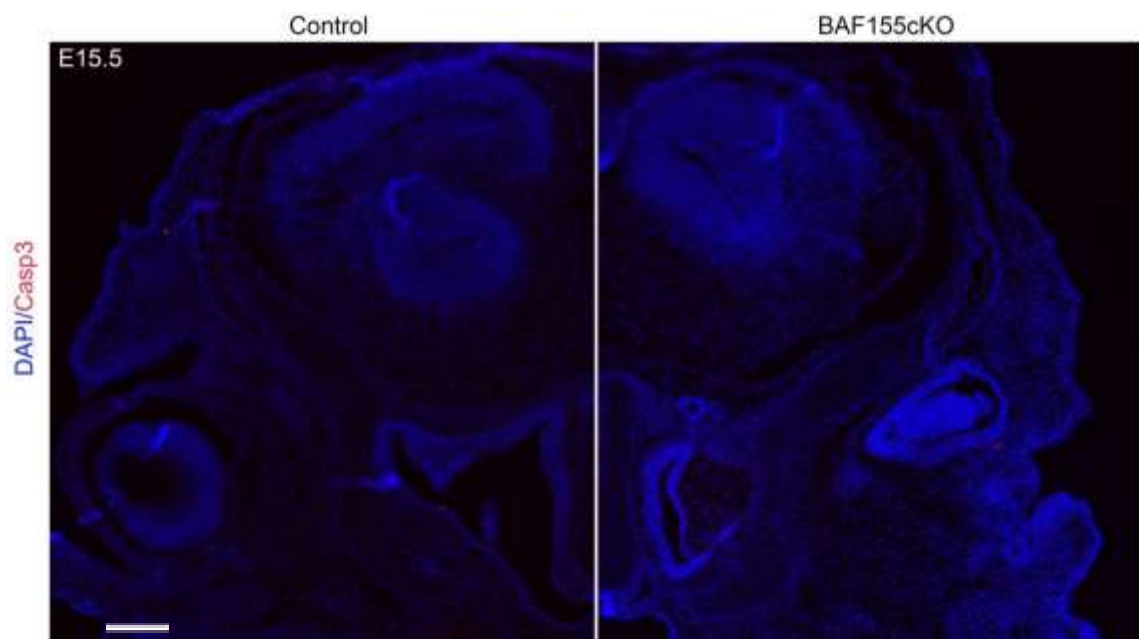


Figure 21: No increased apoptosis in BAF155cKO OE at E15.5

IHC staining of a coronal head section at E15.5 with anti-active caspase3 antibodies for apoptotic cells. The control OE and as well the BAF155cKO mutant only exhibit a few caspase3⁺ cells. Scale bar = 150 μ m.

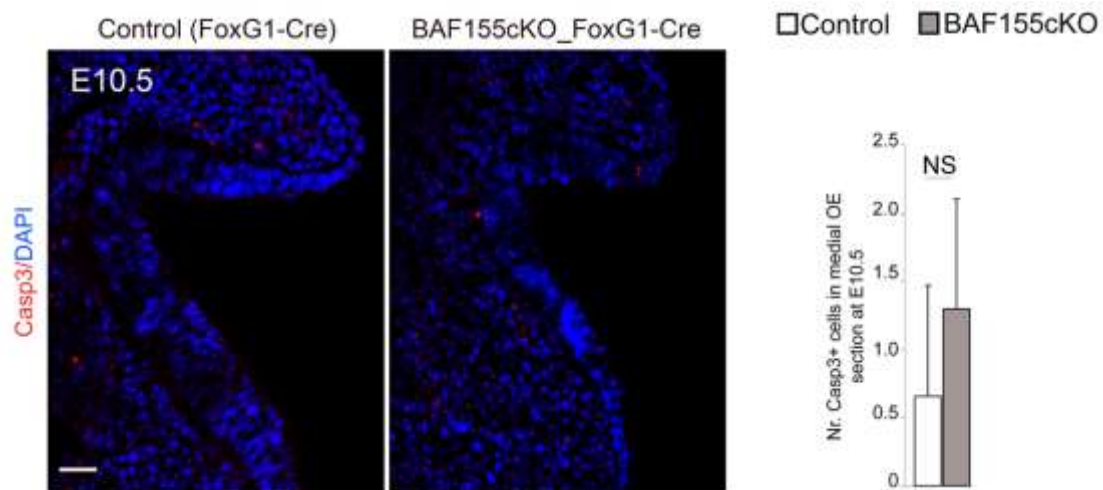


Figure 22: No fundamentally expanded apoptosis in BAF155cKO mutant at E10.5

IHC staining of a sagittal sectioned OE of a control and a mutant mouse reveal few Casp3⁺ apoptotic cells. The panel on the right evidences the not fundamentally expanded number of apoptotic cells in the mutant OE. Scale bar = 25 μ m. Values are reported as means \pm SEM, NS: not significant.

3.7 Loss of pHH3⁺ cells in BAF155cKO embryo: Less mitosis in mutants

The finding that apoptosis is not responsible for the malformation and delayed development of BAF155cKO mutants, led us to examine the cell proliferation by IHC. The M- and late G2-phase specific marker Anti-phospho-Histone H3 (pHH3) (Veras et al. 2009) has been applied on tissue from E10.5, E11.5 and E13.5.

At E10.5 and E11.5, the pHH3 staining is mainly bound to the apical layer of the OE in control. Whereas in the basal layer only few pHH3⁺ mitotic cells in the M-phase are to be found (Figure 23A).

In order to give an exact quantitative result, we made a 3D-image of the OE and counted the pHH3⁺ cells in wild-type and BAF155cKO animals at E10.5, E11.5 and E13.5 (Bachmann et al. 2016). The resulting data makes transparent that the deletion of BAF155 caused a severe decrease in the amount of pHH3⁺ cells in OE at E10.5 and only a slight decrease in number at E11.5 (Figure 23B).

At E13.5 this reduction of mitotic cells can only be noticed in the basal layer (Figure 23B). This decrease of cells in the basal department of the mutant might be due to the fact that oNSCs move towards the basal layer to settle there from E13.5 on. As well the mitotic IPs remain in the basal lamina (Beites et al. 2005).

Figure 23C reveals that the double-stained pHH3/Pax6⁺ stem cells in the mutant are reduced to 40% relative to control. On the contrary, the pHH3/Mash1⁺ intermediate progenitors were not diminished in mutants (Figure 23D).

The pHH3⁺ cells residing in the apical layer are sustentacular cells and are not diminished in the BAF155cKO compared to the control (Figure 23B).

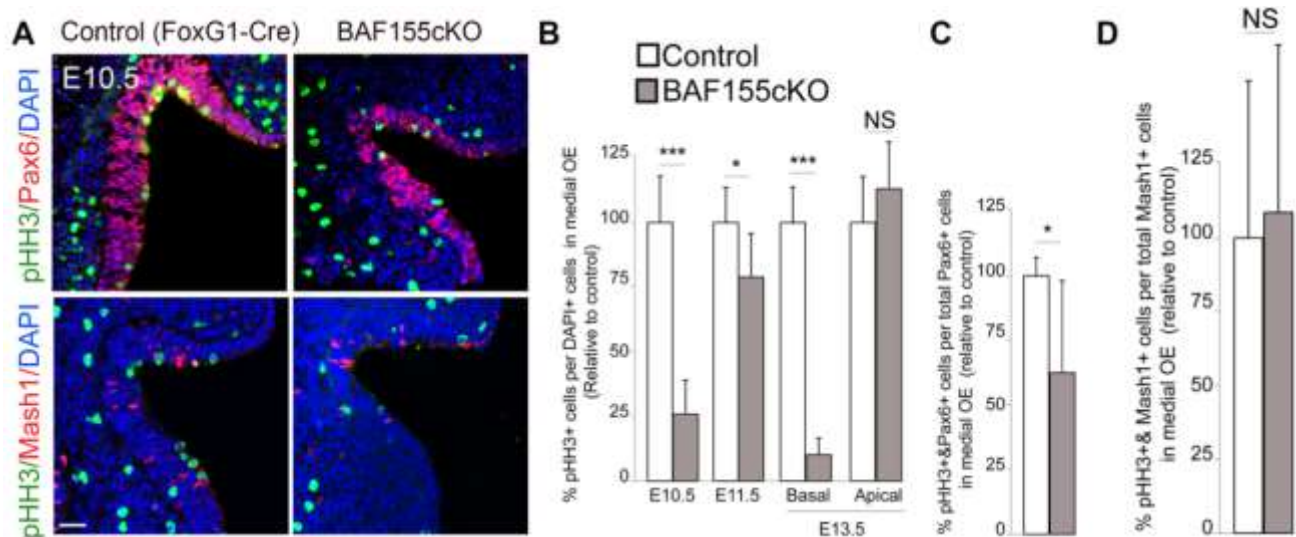


Figure 23: IHC analyses with the mitosis marker pHH3 reveal particular defects in BAF155cKO mutants

A Control and BAF155cKO OE at E10.5 was double-immunostained with Pax6/pHH3 for stem cells and Mash1/pHH3 for IPs and the marker of mitotic cells pHH3. **B** Statistical evaluation reveals, that the knockout of BAF155 causes a punctual decrease of pHH3⁺ cells in early stages (E10.5-E11.5). A decrease of Pax6⁺ OSNs at E10.5 and basal pHH3⁺ oNSCs at E13.5, but not of apical pHH3⁺ SUS cells. **C** Statistical evaluation further reveals that the percentage of pHH3 and Pax6⁺ cells per total Pax6⁺ cells is reduced in the mutant. **(D)** No decrease in pHH3/Mash1⁺ IPs is detectable. Scale bar = 25 μ m. Values are reported as means \pm SEM (* P < 0.05, ** P < 0.01, *** P < 0.001), NS: not significant.

3.8 BAF155cKO mutants have normal olfactory bulb induction but lack the OB outgrowth process

In order to investigate whether the BAF155cKO mutants have a problem with the induction of the olfactory bulb, stainings at E18.5 with a control and a BAF155cKO mouse with Reelin and NP1 have been run. Both markers are quite widely expressed in the region of the forebrain (Okuyama-Yamamoto et al. 2005) including the OB, making it possible to be visualized.

At E18.5, the induction of the OB in the control and BAF155^{-/-} mouse was traceable by Reelin (Figure 24) and NP1 (Figure 24).

Although NP-1⁺ and Reelin⁺ cells in the forebrain region (arrows) give evidence for normal OB induction in the control and in the mutant, the subsequent pro-

cess of OB outgrowth did not occur in the BAF155^{-/-} animal, hence revealing a rudimentary OB structure at a late developmental stage.

The process of outgrowing in the control, however, did physiologically take place (Figure 24, arrows).

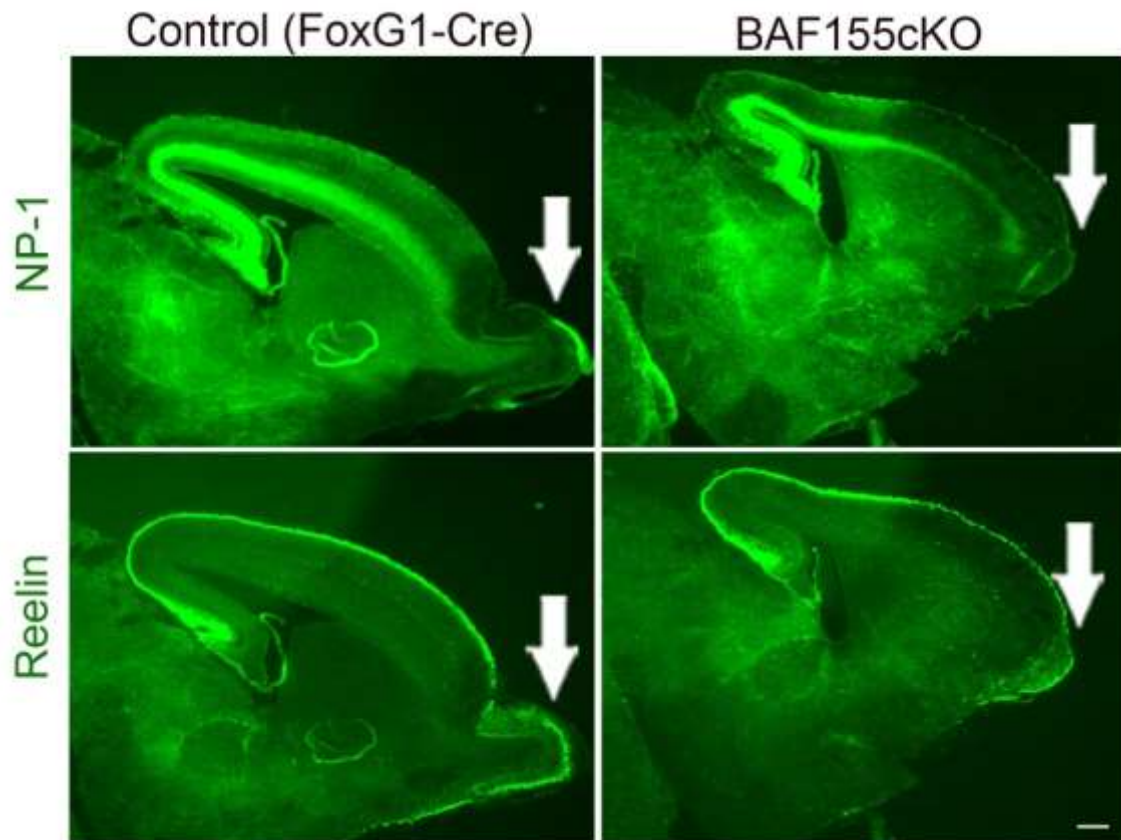


Figure 24: Disrupted outgrowth of the OB in BAF155cKO mutants in the presence of inductive signals

Sagittal head sections of control and BAF155cKO mutant at E18.5 and IHC stainings with Reelin⁺ and NP1⁺ mitral cells. In control, a properly developed OB (arrow) is seen, whereas the OB of the mutant is only rudimentarily developed. Nevertheless, the mutant reveals NP1 and Reelin⁺ mitral cells in the area of the OB (arrow). Scale bar = 100 μ m.

3.9 Defect connection between OE and forebrain at E13.5 and E15.5 in the BAF155cKO animal

Due to the fact that ORNs of the developing OE send their pioneer axons towards the OB, they make direct connection between OE and the telencephalon (Gong and Shipley 1995). To examine the connection between OE and OB, which arises from the forebrain region, we stained tissue from E13.5 and E15.5 with Tuj (Figure 25 & Figure 26) and at E15.5 with N-CAM (Figure 26). The Tuj and N-CAM marked axons of immature OSNs (Terkelsen et al. 1989; Hirata et

al. 2006) are traversing the cribriform plate to connect with the forebrain in the control animal (Figure 25 & Figure 26, arrows). It is essential to emphasize that in the controls the ORN axons and cellular aggregates were positive for Tuj1 and N-CAM immunostaining (Terkelsen et al. 1989; Schwanzel-Fukuda et al. 1992; Smythies and Bradley 1992; Ikeda et al. 2007). However, in the BAF155cKO OE at E13.5 and E15.5 it becomes obvious, that this Tuj⁺ and N-CAM⁺ axonal pathway and formation of cellular aggregates between OE and telencephalon, respectively olfactory bulb does not exist (Figure 25 & Figure 26). Furthermore, we did axon-immunostaining with OMP at E15.5 (Figure 20B). OMP is a marker for mature migratory cells that exit the olfactory placode and migrate with the OSN axons towards the forebrain (Hirata et al. 2006; Menini 2010). Experiments with OMP at E15.5 showed that the control tissue features OMP⁺ migratory cells between the OE and the developing OB in the forebrain region. Conversely, the mutant tissue lacks this axonal connection (Figure 20B).

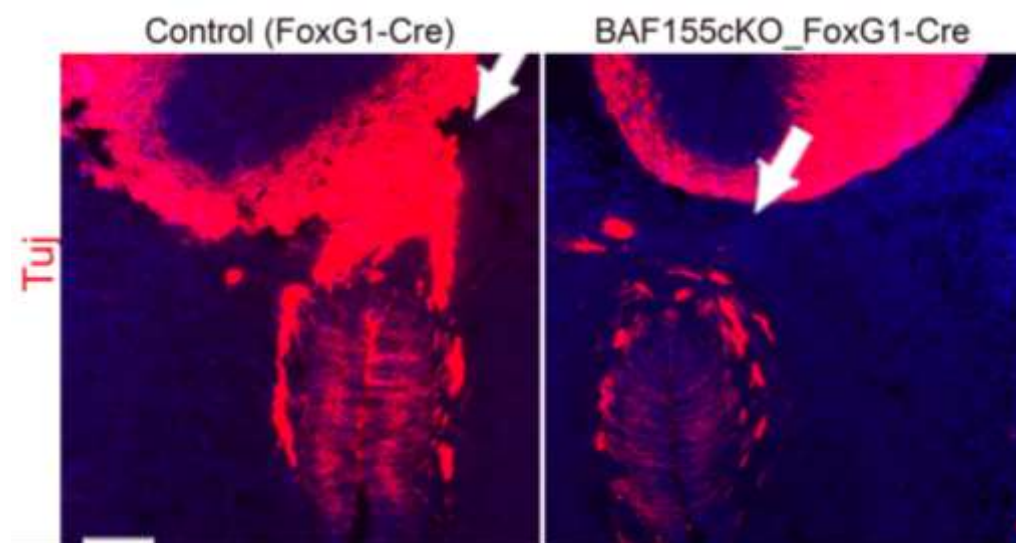


Figure 25: No cellular aggregates and axonal connection between OE and OB in the BAF155cKO mutant

Immunostaining with anti-Tuj antibodies for immature OSNs and their axons of control and BAF155cKO OE at E13.5. Notice a missing axonal connection between OE and forebrain in the mutant. Also, no cellular aggregates as visible in the control animal are to be traced (arrow). Scale bar = 100 μ m.

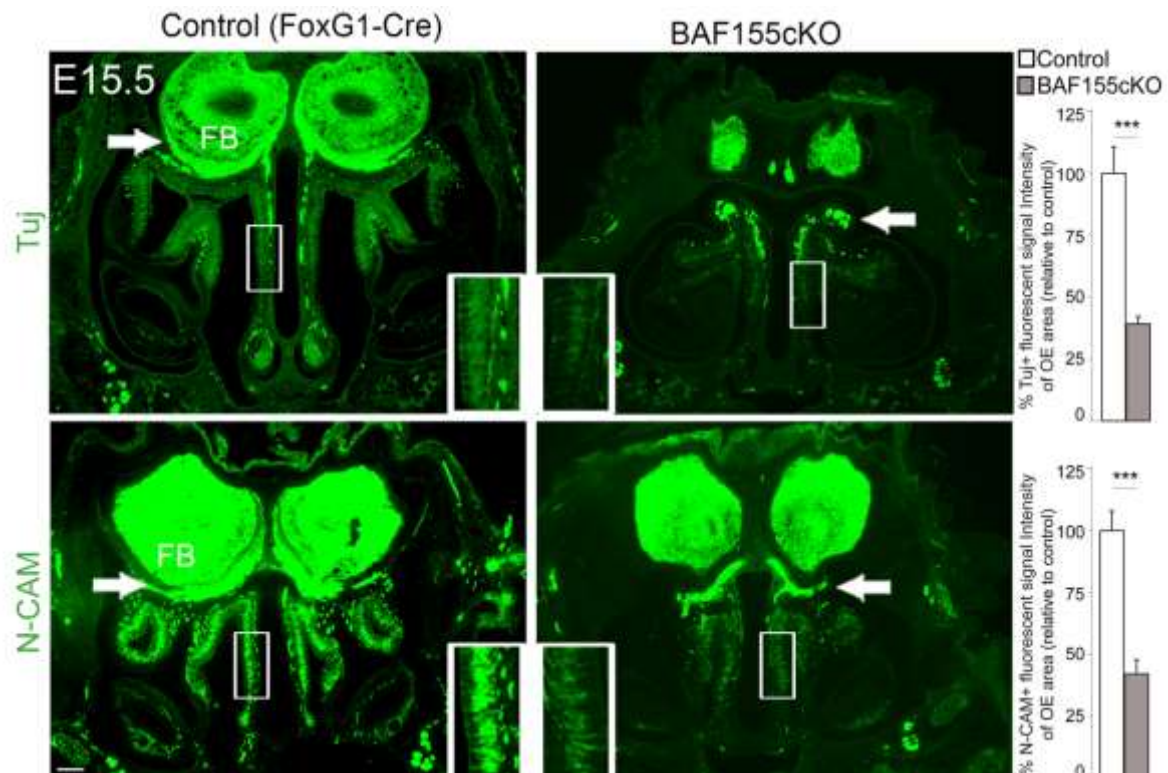


Figure 26: BAF155 is indispensable for the establishment of axonal projections to the forebrain

Immunostaining of coronal head-sections with anti-Tuj antibodies and anti-N-CAM antibodies at E15.5 in wild-type and BAF155cKO mutant. In the control, the bundle of ORN axons and cellular aggregates were positive for Tuj and N-CAM. Contrarily, in BAF155 mutants no Tuj and N-CAM positive axonal patterning is visible between OE and forebrain (arrow). The statistical evaluation reveals a clear diminution of N-CAM and Tuj + cellular aggregates in the BAF155 mutant compared to the control animal (FB: forebrain). Scale bar = 150 μ m.

3.10 Missing sensory input of anterior telencephalic cells and lack of lateral olfactory tract formation

Olfactory sensory neurons residing in the OE project their axons to mitral and tufted cells, that constitute the projection neurons of the OB (Huilgol and Tole 2016). Axogenesis of these mitral cells begins proximately to their ultimate differentiation at around E11.5 (López-Mascaraque et al. 1996; Treloar et al. 2010). From E15 on, physiologically, a solid bundle of fibers can be labeled (López-Mascaraque et al. 1996). Mitral and tufted cells subsequently extend their axonal extensions via the lateral olfactory tract to the primary olfactory cortex. The LOT is situated laterally, among the pia mater (López-Mascaraque et al. 1996; Treloar et al. 2010). We extended our investigations to the question, if anterior telencephalic mitral and tufted cells of BAF155cKO mutants obtain a sensory input from ORNs of the OE. With the aim to trace this axonal connec-

tion between OE, OB and further to the sensory cortex, we injected Dil-crystal into posterior parts of the OE, respectively the nasal cavity at E17.5 in control and BAF155^{-/-} mutants. In the controls, this connection was revealed by fluorescence microscopy (Figure 27) in sagittal head sections. It is apparent that the Dil- labeled axonal bundle in the control animal extends from the OE to the OB, where it synapses with OB neurons. Likewise, the LOT which extends from OB to the olfactory cortex was visualized by Dil⁺ axons in the control (Figure 27). However, in the BAF155 mutant, these projections are completely missing (Figure 27). These pieces of information signify, that BAF155 mutants reveal an impaired development of ORNs to mature and sufficient cells that results in a defect axonal connection to the telencephalon and OB.

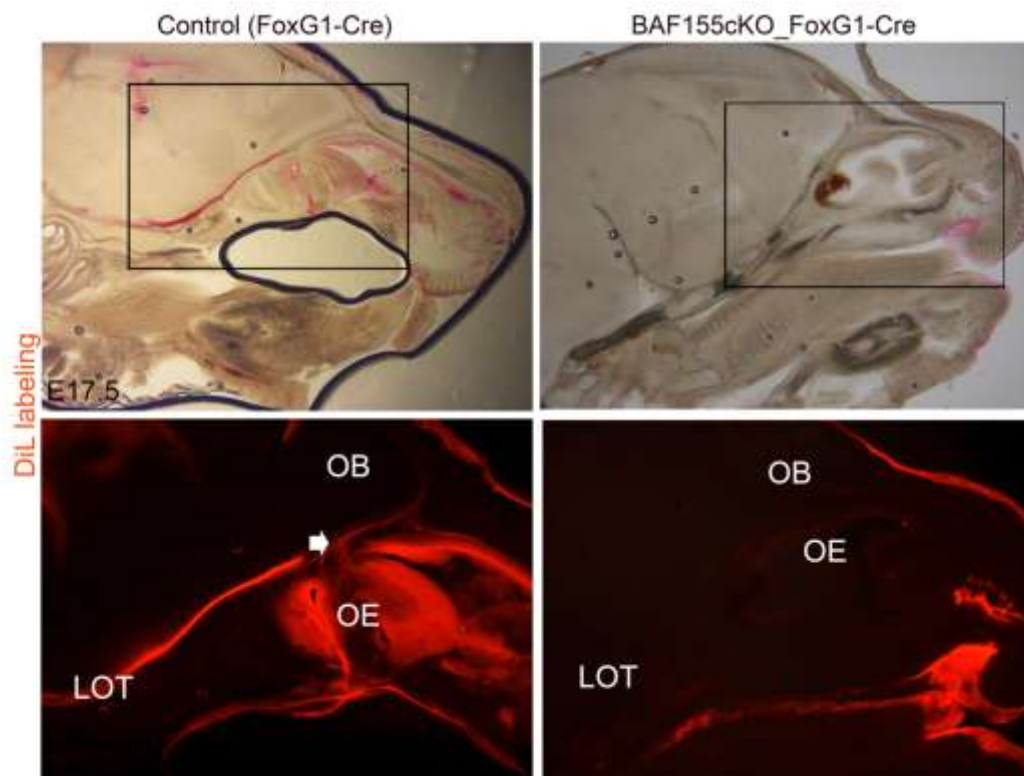


Figure 27: Tracing the olfactory nerve by means of Dil labeling at E17.5 in sagittal head sections of wild-type and BAF155cKO mutant

In the tissue of the control animal the axonal pattern of the olfactory nerve can be traced from OE and OB, respectively from OB neurons (arrow) to the olfactory cortex via the lateral olfactory tract (outlined by a box in the upper panels). Note, that no Dil-positive axonal pathway can be detected in the mutant. **In collaboration with Ahmed Mansouri and Tamara Raabe, Max-Planck-Institute Göttingen**

4 Discussion

The intention of my dissertation project was to define the role of the scaffolding mSWI/SNF (BAF) complex subunit BAF155 in the neurogenic development of the olfactory system of mice. Having in mind, that an advance in understanding the transcriptional network of this self-renewing system can lead to innovative ways to work with stem cells in the field of neurogenerative medicine (Im and Moon 2015).

Considering the good accessibility to the olfactory epithelium and its regenerative qualities, it serves as an outstandingly suitable tissue to investigate the processing of oNSCs.

As in cortical neurogenesis the importance of chromatin regulating BAF complexes has recently been evidenced (Narayanan and Tuoc 2014; Narayanan et al. 2015a; Nguyen et al. 2016), the question arose, in which way the deletion of the scaffolding subunit BAF155 affects the developmental fate of olfactory neural stem cells with regard to the unfolding of their neuronal and non-neuronal cell lineages.

Former investigations with knockout mice have turned out to be successful by making use of immunohistochemistry. Suitably, the stepwise differentiation of oNSCs can be visualized by molecular markers.

The first fundamental finding of my immunohistochemical tracings is, that BAF155 reveals a ubiquitous expression pattern throughout the olfactory cell-lineage (Figure 7 & Figure 8). Accordingly, we detected BAF155 in Pax6⁺, Sox2⁺ neural stem cells, as well in ki67⁺ proliferating progenitors and Mash1⁺ neuronal intermediate progenitors, which are derived from oNSCs. The same applies to the next developmental level of Tuj⁺, HuCD⁺ and LHX⁺ immature neurons, which are capable of proceeding to mature OMP⁺ and Ctip2⁺ olfactory receptor neurons. In the apical layers of olfactory tissue, proliferative glia-like sustentacular cells can be found. This universal expression of BAF155 leads to the assumption that it is of a certain level of importance for the neurogenic activity of oNSCs.

Being aware of this continual existence of BAF155 in the olfactory cell lineages, we next analyzed the phenotype of the BAF155 single mutant (cKO) during development of the OE (Bachmann et al. 2016).

The deletion of BAF155 by means of the Cre-loxP recombination system results in a macroscopic loss of the olfactory bulb at day E18.5 (Figure 5), which normally by E15 should have been formed (Gong and Shipley 1995).

As initially described, the formation of the olfactory bulb can be classified into two developmental processes- first induction, later outgrowth (Gong and Shipley 1995; Besse et al. 2011). These results reveal, that the process of induction takes place, but not the outgrowing (Figure 24).

We found, that mutant mice are lacking the pioneer axons of ORNs, marked by OMP (Menini 2010), Tuj and N-CAM (Figure 20, Figure 25, Figure 26) (Bachmann et al. 2016), that originate from the olfactory epithelium and connect to the axons of mitral cells in the forebrain region between E13.0 and E14.0 (Treloar et al. 2010).

Subsuming these findings, BAF155cKO mice are found to have a deficit in axonal branching between OE and rostral cortex (Bachmann et al. 2016).

This finding further admits the conclusion, that BAF155 is indispensable for the axonal outgrowth of ORNs that extend towards the forebrain. It can be concluded that the disrupted axonal formation of OSNs in the BAF155cKO phenotype and the accompanying lack of connection to mitral cells causes the absence of the outgrowing-process of the olfactory bulb. However, against the odds and despite missing axonal connection, we were able to trace inductional signals in the forebrain region by obtaining positive signals of Reelin and NP1 at E18.5 (Figure 24) (Bachmann et al. 2016). The main source of Reelin expressing cells are the mitral cells in the OB (Hellwig et al. 2012). Reelin as an extracellular matrix glycoprotein (EMC), is able to stick to postsynaptic cells' surface receptors, to subsequently cause intracellular cascades. Diverse studies propose that Reelin and its receptor apoER2 are involved in synaptic plasticity (Dityatev and Schachner 2006).

The finding that we traced inductional signals in mutant mice, despite missing axonal connections, contradicts early reports in which the appearance of olfactory axons in the ventricular zone of the telencephalon was linked with the process of induction (Gong and Shipley 1995).

The induction of the olfactory bulb maturation for them accordingly correlates with the presence of pioneer olfactory axons in the area of the ventricular zone by modifying cell cycle kinetics.

However, they do not exclude the alternative, that the alterations in cell cycle kinetics are due to inherent processes of the olfactory primordium (Gong and Shipley 1995).

Our results are even more in conformance with the investigations of Besse et al, who stated that the absence of connection between mitral cells and OSNs axons might occupy a vital role in the OB development (Besse et al. 2011).

In any case, the question if the normal processing of the OB is up to axonal connections of ORNs, is to date in the focus of research (Gong and Shipley 1995; Jiménez et al. 2000; Hirata et al. 2006; Besse et al. 2011; Bachmann et al. 2016) and a careful analysis of the precise course of the inductional process might be meaningful.

We expanded the investigations of axonal connections in the BAF155cKO phenotype to the subsequent axonal projections of mitral cells to the primary olfactory cortex. Projection neurons of mitral cells physiologically form the lateral olfactory tract, which establishes a connection to the olfactory cortex. This axonal outspreading process normally takes place at E11 - E13, shortly before OSN axons reach the OB at E14 (Blanchart et al. 2006).

To observe the axonal connection and extension, Dil crystal was injected into the OE in the posterior section of the nasal cavity in control and mutant at E17.5 and the sagittal sections were analyzed (Bachmann et al. 2016). A clear Dil-labeled bunch of axons can be visualized in the control animal at E17.5 between OE and OB, and further via the LOT to the primary olfactory cortex (Figure 27).

The finding, however, that in the mutant animal no axonal projection pattern is visible between OE and OB, as well not between OB and olfactory cortex, terminates in the interpretation of a disrupted axonal connection of the BAF155cKO mutant in the primary and as well in the secondary olfactory pathway.

In the course of investigations, it became apparent that the BAF155 subunit is indispensable to the formation of oNSCs, as the deletion of BAF155 terminates in a severe reduction of oNSCs in early neurogenesis (Figure 8). Interestingly,

prevailing studies which focused on BAF155 in cortical tissue have also exposed that BAF155 is increasingly measured in dividing stem/progenitor cells (Nguyen et al. 2016). It is thus conceivable, that the self-renewal and the proliferative potential of olfactory stem cells is dependent on a sufficient BAF complex (Bachmann et al. 2016).

The loss in the pool of stem cells consequently leads to a progressive degradation of the apical progenitor pool in late neurogenesis, however, the early neuronal specification from oNSC to immature ORN seems to be preserved.

Accordingly, the generation of intermediate progenitors and immature neurons in the OE at the early developmental stage of E10.5 appears unaffected due to the slight reduction of the progenitor specific marker Mash1 (Figure 11) and pioneer specific marker Tuj (Figure 12). The marker HuCD for pioneer neurons is even upregulated at E10.5 in mutant mice (Figure 11). Hence, it is important to emphasize that BAF155 does not occupy a key function in the early processing from OSN to ORN.

As mentioned before, the intermediate progenitor pool in later neurogenesis, a stadium of proliferation and differentiation, is severely reduced in number.

The intermediate progenitor cells, marked by Mash1 exhibit a reduction of about 75% at E13.5 (Figure 16), likewise, (HuCD⁺) immature ORNs are decreased to 50% (Figure 17). My findings are consistent with previous studies, which reflect the importance of BAF complexes in controlling the transcription of genes participating in pluripotency and self-regeneration of progenitors. These studies supply evidence that mice heterozygous for BAF155 are prone to exencephaly, presumably due to the generation of insufficient neurons, traced back as well to defects in neural progenitor self-regeneration and differentiation (Bultman et al. 2000; Kim et al. 2001a; Ho et al. 2009; Panamarova et al. 2016).

The analysis of the subsequent cellular level of mature ORNs comes out with a clear decrease of mature OMP⁺ and Ctip2⁺ cells.

Immunohistochemical stainings have been implemented at day E10.5, E11.5, E13.5 and day E15.5. At all developmental levels, the amount of mature ORNs is severely reduced in the mutant OE compared to the wild-type OE (Figure 19 & Figure 20).

These results imply that despite the fact that early neurogenesis is not disturbed, the loss of BAF155 results in a disturbance of neurogenesis at later OE

developmental stages (Bachmann et al. 2016). According to Beites et al (2015), proneural gene-function like Mash1 in IPs, is indispensable for the differentiation of oNSCs to ORN, whilst suppressing the non-neuronal SUS formation (Beites et al. 2005; Im and Moon 2015). In other words, Mash1 may be a determining factor in the decision of whether the IPs develop into neurons or SUS cells (Murray et al. 2003). This finding supports the subsequent reduction of HuCD⁺ ORNs after the decline of Mash1⁺ progenitors in BAF155cKO mutants in late neurogenesis (Figure 16). Since Mash1 is a determinant of the neuronal lineage, it may be the logical consequence, that the non-neuronal SUS-pathway is upregulated or affected in the absence of Mash1.

As previously mentioned, NSCs in the OE can generate ORNs and sustentacular cells. These sustentacular cells inherit glial-like functions, for instance formative and metabolic supply (Schwob 2002; Gokoffski et al. 2011; Kam et al. 2014).

The careful investigation of the sustentacular cell fate indicated, that the quantitative outcome is unaffected by the loss of BAF155 (Figure 13 & Figure 14).

In order to confirm the upcoming thesis, that the knockout of BAF155 perturbs the proliferation of only oNSCs, and late IPs, but not the proliferation of IPs in early neurogenesis and SUS cells, we performed immunohistochemical stainings with pHH3. This proliferation marker is positive for cells, which are situated in M-Phase of mitosis.

The evaluation indeed affirms that in comparison to controls, the ratio of pHH3⁺/Pax6⁺ proliferative stem cells (Figure 23A & Figure 23C), however, not of pHH3⁺/Mash1⁺ intermediate progenitor cells in mutants was lower at E10.5 (Figure 23A & Figure 23D). The staining with pHH3 at day E13.5 reveals that the reduction of cells is restricted to the basally located stem cells (Figure 23B). The explanation for this outcome might be that oNSCs move to reside inside the basal lamina from day E13.5 on.

To review the pool of dividing SUS cells in the apical region, it is obvious that their proliferation as supposed remains unaffected (Figure 23B). This finding leads to the postulation that BAF155 is not a universal modulator of cellular proliferation. It selectively interferes with the proliferation of oNSCs, but not the development of intermediate neuronal progenitors in early neurogenesis and dividing SUS cells (Bachmann et al. 2016).

It has to be raised the apparent question, why the early neurogenesis and proliferation of SUS cells is unaffected by a dysfunction of BAF155. The unimpaired generation of SUS may be due to the fact, that BAF155-activity mainly affects pluripotent cells. Interestingly, even the subunit Brg is not essential for the proliferation of fibroblasts or glia, but is indispensable for the multipotency of neural stem cells (Wu et al. 2009). A further explanation might be the before mentioned downregulation of Mash1⁺ cells and the therewith associated lack of proneural gene-function in BAF155cKO mice.

To draw a striking parallel to the human olfactory epithelium, it is interesting to mention, that in the process of aging, one of two pathologic manifestations can be the disappearance of OSNs, while SUS cells and HBCs (horizontal basal cells) remain (Schwob et al. 2017). It is important to emphasize that the regeneration of the OE incorporates distinct stages. Namely, the proliferation of progenitors, the already mentioned cell-fate determination between neuronal and non-neuronal paths to SUS, in which Mash1 plays a primary role, and their consequent unfolding (Im and Moon 2015).

Nevertheless, a complete perception of the potential differentiation of oNSCs into SUS necessitates further investigations (Bachmann et al. 2016).

In this context, it would be meaningful to examine the part of BAF155 in terms of cell fate calculation by means of fate tracing with an oNSC-specific Cre-line. Figure 9 illustrates, that the above-mentioned downregulation of oNSCs, late IPs and mature ORNs, mirrors in a discrepancy in surface dimension of the olfactory epithelium between control and mutant. Interestingly, this discrepancy constantly amplifies from E10.5 to E15.5. As that, BAF155cKO mice reveal a delayed state of development with regard to the constitution of the olfactory epithelium.

At E11.5 the invagination process from olfactory placode to nasal cavity should already have taken place and the openings of the nasal pits should have been constricted to form the nares. However, the mutant phenotype reveals a structure which still remains a cuplike morphology which is characteristic for E 10.5 (Menini 2010) (Figure 9A & Figure 9B). BAF155 is thus apparently central to the self-renewal and proliferation of oNSCs in the olfactory epithelium.

Interestingly, former studies with cortex-specific BAF170cKO mice, another scaffolding subunit of the BAF complex, generated similar phenotypes with re-

duced cerebral cortical volume and thickness. Even BAF170 seems to control IP differentiation (Tuoc et al. 2013b; Narayanan and Tuoc 2014).

For the purpose of excluding the possibility to find the cause of the reduced cell pool in mutants rather in apoptosis than in disturbed proliferation, final stainings have been performed with the apoptosis marker Caspase3. The collected data reveal, that the control and as well the BAF155^{-/-} mouse OE only exhibit few Casp3⁺ apoptotic cells (Figure 21 & Figure 22). Thus, BAF155 cKO mice exhibit no increased apoptotic activity, as shown by IHC.

The presented results verify the ubiquitous existence of BAF155 in the cell lineage of the olfactory epithelium. Furthermore, the data reveals that BAF155 is apparently central to the proliferation of oNSCs and late IPs and associated with a defect in the maturation and axogenesis of OSNs. Nevertheless, BAF155 is not a universal modulator of olfactory cellular proliferation.

5 Abstract

Neurogenesis is a core developmental process which comprises the maturation of neural stem cells to fully developed neurons. Former studies have provided evidence that in the neuronal development of the central nervous system, chromatin remodeling mSWI/SNF (BAF) complexes are indispensable factors to guide the proliferation and renewal of cells.

Accordingly, given the key role in the central nervous system, the BAF complex currently receives scientific attention in how it regulates neurogenesis.

As the olfactory epithelium constitutes an exceptional tissue, characterized by self-renewing capacities throughout life, it serves as an excellent model to explore how neurogenesis is controlled at cellular and molecular levels (Kawauchi 2005).

Thus, this study sheds light on the role of the scaffolding subunit BAF155 in the development of the olfactory system of mice.

It must be highlighted that BAF155 is ubiquitously expressed in the neuronal and non-neuronal cell lineages of the olfactory epithelium. On the basis of this finding, I analyzed the phenotype of a BAF155 conditional knockout mutant by means of immunohistochemical tracings in the olfactory epithelium of mice.

The BAF155cKO phenotype is characterized by a macroscopic loss of the olfactory bulb despite inductive signals in the corresponding region of the fore-brain, an impaired proliferation of the oNSC population and a defect in the maturation of ORNs which results in a thinner OE and as well in a loss of axonal projections to higher brain regions. However, BAF155 is no universal modulator of olfactory cellular proliferation, as the population of glial-like sustentacular cells is totally preserved in the BAF155cKO mutant. Furthermore, the early neuronal specification around E10.5 from oNSC to immature ORN seems to be preserved.

Further studies may be acquired in order to complete our comprehension of the processing of oNSCs into sustentacular cells and why this developmental pathway is not affected by a loss of BAF155. Closer investigations should also be invested in sorting out, whether the development of the olfactory bulb is conditional upon physiologic axonal connections between OE and OB.

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7 Attachment

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Studientitel: Roles of BAF 155 in the development of the olfactory systems

Antragsteller: Dr. med. Joachim Rosenbusch, Institut für Neuroanatomie, UMG

Doktorandin: Christina Bachman

Sehr geehrte Frau Bachmann, sehr geehrter Herr Dr. Rosenbusch,

bei dem hier vorgelegten und unter unserer oben genannten Nummer in der Ethik-Kommission archivierten Promotionsverfahren handelt es sich nach allen vorliegenden Informationen ausschließlich um Tierversuche.

Die Ethik-Kommission ist nicht zuständig für Tierversuche. Wissenschaftliche Arbeiten und entsprechend natürlich auch Doktorarbeiten, bei denen es ausschließlich um Tierversuche geht, brauchen unserer Ethikkommission, die für medizinische Forschung am Menschen zuständig ist, nicht vorgelegt zu werden.

Nur allgemein rein vorsorglich weisen wir darauf hin, dass vorliegendes Schreiben Sie natürlich nicht davon entbindet, gegebenenfalls einen Tierversuchsantrag bei der zuständigen Behörde zu stellen und dass es bezüglich unserer Forschungs-Ethikkommission eine Beratungspflicht geben kann, wenn in den Tiermodellen humanes Biomaterial (z.B. humanes Tumorgewebe) eingebracht wird. Dann wird dafür in der Regel sowohl eine Genehmigung der zuständigen Tierschutzbehörde als auch ein Votum der Ethikkommission erforderlich sein. Derartiges humanes Biomaterial scheint bei vorliegendem Projekt aber nicht relevant zu sein.

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